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APPLICATION FOR UNITED STATES PATENT

CIRCULAR PERMUTEINS OF FLT3 LIGAND

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CIRCULAR PERMUTEINS OF FLT3 LIGAND

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] The present application is a divisional of U.S. Pat. App. Ser. No. 08/955,090, filed October 21, 1997, pending; which claims priority under Title 35, United States Code, § 119(e)(1) of U.S. Prov. Pat. App. Ser. No. 60/030,094, filed October 25, 1996.

FIELD OF THE INVENTION

[002] The present invention relates to human flt3 receptor agonists. These flt3 receptor agonists retain one or more activities of native flt3 ligand and may also show improved hematopoietic cell-stimulating activity and/or an improved activity profile which may include reduction of undesirable biological activities associated with native flt3 ligand and/or have improved physical properties which may include increased solubility, stability and refold efficiency.

REFERENCE TO A "SEQUENTIAL LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED ON A COMPACT DISC

[003] This application includes a computer program listing appendix, pursuant to 37 CFR 1.96, contained on a compact disc,

which is incorporated fully into this application by this reference.

The compact disc is labeled as follows:

Inventors: Charles A. McWherter, John P. McKearn, Philip R. Streeter, Nancy I. Minster, Yiquing Feng, Nicholas R. Staten, Susan L. Woulfe, & John C. Minnerly
Title: Circular Permuteins of FLT3 Ligand
Attorney docket number: 126181-1059
Creation date of the compact disc: August 20, 2003

The compact disc contains the following files in ASCII file format:

File Name	**File size**	**Creation Date**
Seq List 2993-3 US.txt	118 kb	August 20, 2003

BACKGROUND OF THE INVENTION

[004] Colony stimulating factors which stimulate the differentiation and/or proliferation of bone marrow cells have generated much interest because of their therapeutic potential for restoring depressed levels of hematopoietic stem cell-derived cells. Colony stimulating factors in both human and murine systems have been identified and distinguished according to their activities. For example, granulocyte-CSF (G-CSF) and macrophage-CSF (M-CSF) stimulate the in vitro formation of neutrophilic granulocyte and macrophage colonies, respectively while GM-CSF and interleukin-3 (IL-3) have broader activities and stimulate

the formation of both macrophage, neutrophilic and eosinophilic granulocyte colonies. Certain factors such as flt3 ligand are able to predominately affect stem cells.

[005] Tyrosine kinase receptors are growth factor receptors that regulate the proliferation and differentiation of a number of cell. Certain tyrosine kinase receptors function within the hematopoietic system. Flt3 ligand (Rosnet et al., *Oncogene*, **6**:1641-1650, 1991) and flk-2 (Matthews et al., *Cell*, **65**:1143-1152, 1991) are forms of a tyrosine kinase receptor that is related to c-fms and c-kit receptors. The flk-2 and flt3 receptors are similar in amino acid sequence and vary at two amino acid residues in the extracellular domain and diverge in a 31 amino acid segment located near the C-terminus.

[006] flt3 ligand is a hematopoietic growth factor which has the property of being able to regulate the growth and differentiation of hematopoietic progenitor and stem cells. Because of its ability to support the growth and proliferation of progenitor cells, flt3 receptor agonists have potential for therapeutic use in treating hematopoietic disorders such as aplastic anemia and myelodysplastic syndromes. Additionally, flt3 receptor agonists will be useful in restoring hematopoietic cells to normal amounts in those cases where the number of cells has

been reduced due to diseases or to therapeutic treatments such as radiation and chemotherapy.

[007] WO 94/28391 discloses the native flt3 ligand protein sequence and a cDNA sequence encoding the flt3 ligand, methods of expressing flt3 ligand in a host cell transfected with the cDNA and methods of treating patients with a hematopoietic disorder using flt3 ligand.

[008] US Patent No. 5,554,512 is directed to human flt3 ligand as an isolated protein, DNA encoding the flt3 ligand, host cells transfected with cDNAs encoding flt3 ligand and methods for treating patients with flt3 ligand.

[009] WO 94/26891 provides mammalian flt3 ligands, including an isolate that has an insertion of 29 amino acids, and fragments there of.

Rearrangement of Protein Sequences

[0010] In evolution, rearrangements of DNA sequences serve an important role in generating a diversity of protein structure and function. Gene duplication and exon shuffling provide an important mechanism to rapidly generate diversity and thereby provide organisms with a competitive advantage, especially since the basal mutation rate is low (Doolittle, *Protein Science* **1**:191-200, 1992).

[0011] The development of recombinant DNA methods has made it possible to study the effects of sequence transposition on protein folding, structure and function. The approach used in creating new sequences resembles that of naturally occurring pairs of proteins that are related by linear reorganization of their amino acid sequences (Cunningham, et al., *Proc. Natl. Acad. Sci. U.S.A.* **76**:3218-3222, 1979; Teather & Erfle, *J. Bacteriol.* **172**: 3837-3841, 1990; Schimming et al., *Eur. J. Biochem.* **204**: 13-19, 1992; Yamiuchi and Minamikawa, *FEBS Lett.* **260**:127-130, 1991: MacGregor et al., *FEBS Lett.* **378**:263-266, 1996). The first in vitro application of this type of rearrangement to proteins was described by Goldenberg and Creighton (*J. Mol. Biol.* **165**:407-413, 1983). A new N-terminus is selected at an internal site (breakpoint) of the original sequence, the new sequence having the same order of amino acids as the original from the breakpoint until it reaches an amino acid that is at or near the original C-terminus. At this point the new sequence is joined, either directly or through an additional portion of sequence (linker), to an amino acid that is at or near the original N-terminus, and the new sequence continues with the same sequence as the original until it reaches a point that is at or near the amino acid that was N-terminal to the breakpoint site of the original sequence, this residue forming the new C-terminus of the chain.

[0012] This approach has been applied to proteins which range in size from 58 to 462 amino acids (Goldenberg & Creighton, *J. Mol. Biol.* **165**:407-413, 1983; Li & Coffino, *Mol. Cell. Biol.* **13**:2377-2383, 1993). The proteins examined have represented a broad range of structural classes, including proteins that contain predominantly α -helix (interleukin-4; Kreitman et al., *Cytokine* **7**:311-318, 1995), β -sheet (interleukin-1; Horlick et al., *Protein Eng.* **5**:427-431, 1992), or mixtures of the two (yeast phosphoribosyl anthranilate isomerase; Luger et al., *Science* **243**:206-210, 1989). Broad categories of protein function are represented in these sequence reorganization studies:

Enzymes

T4 lysozyme	Zhang et al., *Biochemistry* **32**:12311-12318 (1993); Zhang et al., *Nature Struct. Biol.* **1**:434-438 (1995)
dihydrofolate reductase	Buchwalder et al., *Biochemistry* **31**:1621-1630 (1994); Protasova et al., *Prot. Eng.* **7**:1373-1377 (1995)
ribonuclease T1	Mullins et al., *J. Am. Chem. Soc.* **116**:5529-5533 (1994); Garrett et al., *Protein Science* **5**:204-211 (1996)
Bacillus β-glucanse	Hahn et al., *Proc. Natl. Acad. Sci. U.S.A.* **91**:10417-10421 (1994)

aspartate transcarbamoylase	Yang & Schachman, *Proc. Natl. Acad. Sci. U.S.A.* **90**:11980-11984 (1993)
phosphoribosyl anthranilate isomerase	Luger et al., *Science* **243**:206-210 (1989); Luger et al., *Prot. Eng.* **3**:249-258 (1990)
pepsin/pepsinogen	Lin et al., *Protein Science* **4**:159-166 (1995)
glyceraldehyde-3-phosphate dehydrogenase	Vignais et al., *Protein Science* **4**:994-1000 (1995)
ornithine decarboxylase	Li & Coffino, *Mol. Cell. Biol.* **13**:2377-2383 (1993)
yeast phosphoglycerate dehydrogenase	Ritco-Vonsovici et al., *Biochemistry* **34**:16543-16551 (1995)

Enzyme Inhibitor

basic pancreatic trypsin inhibitor	Goldenberg & Creighton, *J. Mol. Biol.* **165**:407-413 (1983)

Cytokines

interleukin-1β	Horlick et al., *Protein Eng.* **5**:427-431 (1992)
interleukin-4	Kreitman et al., *Cytokine* **7**:311-318 (1995)

Tyrosine Kinase Recognition Domain

α-spectrin SH3 domain	Viguera, et al., *J. Mol. Biol.* **247**:670-681 (1995)

Transmembrane Protein

omp A	Koebnik & Krämer, *J. Mol. Biol.* **250**:617-626 (1995)

Chimeric Protein

interleukin-4–*Pseudomonas* exotoxin fusion molecule	Kreitman et al., *Proc. Natl. Acad. Sci. U.S.A.* **91**:6889-6893 (1994)

[0013] The results of these studies have been highly variable. In many cases substantially lower activity, solubility or thermodynamic stability were observed (*E. coli* dihydrofolate reductase, aspartate transcarbamoylase, phosphoribosyl anthranilate isomerase, glyceraldehyde-3-phosphate dehydrogenase, ornithine decarboxylase, omp A, yeast phosphoglycerate dehydrogenase). In other cases, the sequence rearranged protein appeared to have many nearly identical properties as its natural counterpart (basic pancreatic trypsin inhibitor, T4 lysozyme, ribonuclease T1, Bacillus β-glucanase, interleukin-1β, α-spectrin SH3 domain, pepsinogen, interleukin-4). In exceptional

cases, an unexpected improvement over some properties of the natural sequence was observed, e.g., the solubility and refolding rate for rearranged α-spectrin SH3 domain sequences, and the receptor affinity and anti-tumor activity of transposed interleukin-4—*Pseudomonas* exotoxin fusion molecule (Kreitman et al., *Proc. Natl. Acad. Sci. U.S.A.* **91**:6889-6893, 1994; Kreitman et al., *Cancer Res.* **55**:3357-3363, 1995).

[0014] The primary motivation for these types of studies has been to study the role of short-range and long-range interactions in protein folding and stability. Sequence rearrangements of this type convert a subset of interactions that are long-range in the original sequence into short-range interactions in the new sequence, and vice versa. The fact that many of these sequence rearrangements are able to attain a conformation with at least some activity is persuasive evidence that protein folding occurs by multiple folding pathways (Viguera, et al., *J. Mol. Biol.* **247**:670-681, 1995). In the case of the SH3 domain of α-spectrin, choosing new termini at locations that corresponded to β -hairpin turns resulted in proteins with slightly less stability, but which were nevertheless able to fold.

[0015] The positions of the internal breakpoints used in the studies cited here are found exclusively on the surface of proteins, and are distributed throughout the linear sequence

without any obvious bias towards the ends or the middle (the variation in the relative distance from the original N-terminus to the breakpoint is ca. 10 to 80% of the total sequence length). The linkers connecting the original N- and C-termini in these studies have ranged from 0 to 9 residues. In one case (Yang & Schachman, *Proc. Natl. Acad. Sci. U.S.A.* **90**:11980-11984, 1993), a portion of sequence has been deleted from the original C-terminal segment, and the connection made from the truncated C-terminus to the original N-terminus. Flexible hydrophilic residues such as Gly and Ser are frequently used in the linkers. Viguera, et al.(*J. Mol. Biol.* **247**:670-681, 1995) compared joining the original N- and C- termini with 3- or 4-residue linkers; the 3-residue linker was less thermodynamically stable. Protasova et al. (*Protein Eng.* **7**:1373-1377, 1994) used 3- or 5-residue linkers in connecting the original N-termini of *E. coli* dihydrofolate reductase; only the 3-residue linker produced protein in good yield.

SUMMARY OF THE INVENTION

[0016] The modified human flt3 receptor agonists of the present invention can be represented by the Formula:

$$X^1-(L)_a-X^2$$

wherein;

a is 0 or 1;

X^1 is a peptide comprising an amino acid sequence corresponding to the sequence of residues n+1 through J;

X^2 is a peptide comprising an amino acid sequence corresponding to the sequence of residues 1 through n;

n is an integer ranging from 1 to J-1; and

L is a linker.

[0017] In the formula above the constituent amino acids residues of human flt3 ligand are numbered sequentially 1 through J from the amino to the carboxyl terminus. A pair of adjacent amino acids within this protein may be numbered n and n+1 respectively where n is an integer ranging from 1 to J-1. The residue n+1 becomes the new N-terminus of the new flt3 receptor agonist and the residue n becomes the new C-terminus of the new flt3 receptor agonist.

[0018] The present invention relates to novel flt3 receptor agonists of the following formula:

```
ThrGlnAspCysSerPheGlnHisSerProIleSerSerAspPheAlaValLysIleArg
                          10                  20

GluLeuSerAspTyrLeuLeuGlnAspTyrProValThrValAlaSerAsnLeuGlnAsp
                          30                  40

GluGluLeuCysGlyGlyLeuTrpArgLeuValLeuAlaGlnArgTrpMetGluArgLeu
                          50                  60

LysThrValAlaGlySerLysMetGlnGlyLeuLeuGluArgValAsnThrGluIleHis
                          70                  80

PheValThrLysCysAlaPheGlnProProProSerCysLeuArgPheValGlnThrAsn
                          90                  100

IleSerArgLeuLeuGlnGluThrSerGluGlnLeuValAlaLeuLysProTrpIleThr
                          110                 120

ArgGlnAsnPheSerArgCysLeuGluLeuGlnCysGlnProAspSerSerThrLeuPro
                          130                 140

ProProTrpSerProArgProLeuGluAlaThrAlaProThrAlaProGlnProProLeu
                          150                 160

LeuLeuLeuLeuLeuLeuProValGlyLeuLeuLeuLeuAlaAlaAlaTrpCysLeuHis
                          170                 180

TrpGlnArgThrArgArgArgThrProArgProGlyGluGlnValProProValProSer
                          190                 200

ProGlnAspLeuLeuLeuValGluHis SEQ ID NO:145
                      209
```

wherein the N-terminus is joined to the C-terminus directly or through a linker capable of joining the N-terminus to the C-terminus and having new C- and N-termini at amino acids;

28-29	42-43	93-94
29-30	64-65	94-95
30-31	65-66	95-96
31-32	66-67	96-97

32-33	86-87	97-98
34-35	87-88	98-99
36-37	88-89	99-100
37-38	89-90	100-101
38-39	90-91	101-102
39-40	91-92	102-103
40-41	92-93	respectively; and
41-42		

additionally said flt3 receptor agonist polypeptide can be immediately preceded by (methionine^{-1}), (alanine^{-1}) or (methionine^{-2}, alanine^{-1}).

[0019] A preferred embodiment is human flt3 receptor agonist polypeptide, comprising a modified flt3 ligand amino acid sequence of the Formula:

```
ThrGlnAspCysSerPheGlnHisSerProIleSerSerAspPheAlaValLysIleArg
                          10                            20

GluLeuSerAspTyrLeuLeuGlnAspTyrProValThrValAlaSerAsnLeuGlnAsp
                          30                            40

GluGluLeuCysGlyGlyLeuTrpArgLeuValLeuAlaGlnArgTrpMetGluArgLeu
                          50                            60

LysThrValAlaGlySerLysMetGlnGlyLeuLeuGluArgValAsnThrGluIleHis
                          70                            80

PheValThrLysCysAlaPheGlnProProProSerCysLeuArgPheValGlnThrAsn
                          90                            100

IleSerArgLeuLeuGlnGluThrSerGluGlnLeuValAlaLeuLysProTrpIleThr
                          110                           120

ArgGlnAsnPheSerArgCysLeuGluLeuGlnCysGlnProAspSerSerThrLeu
                          130             SEQ ID NO:144
```

wherein the N-terminus is joined to the C-terminus directly or through a linker capable of joining the N-terminus to the C-terminus and having new C- and N-termini at amino acids;

28-29	42-43	93-94
29-30	64-65	94-95
30-31	65-66	95-96
31-32	66-67	96-97
32-33	86-87	97-98
34-35	87-88	98-99
36-37	88-89	99-100
37-38	89-90	100-101
38-39	90-91	101-102
39-40	91-92	102-103
40-41	92-93	respectively; and
41-42		

additionally said flt3 receptor agonist polypeptide can be immediately preceded by (methionine^{-1}), (alanine^{-1}) or (methionine^{-2}, alanine^{-1}).

[0020] The more preferred breakpoints at which new C-terminus and N-terminus can be made are 36-37, 37-38, 38-39, 39-40, 40-41, 41-42, 42-43, 64-65, 65-66, 66-67, 86-87, 87-88, 88-89, 89-90, 90-91, 91-92, 92-93, 93-94, 95,-96, 96-97, 97-98, 99-100 and 100-101

[0021] The most preferred breakpoints at which new C-terminus and N-terminus can be made are; 39-40, 65-66, 89-90, 99-100 and 100-101.

[0022] The flt3 receptor agonists of the present invention may contain amino acid substitutions, deletions and/or insertions. It

is also intended that the flt3 receptor agonists of the present invention may also have amino acid deletions at either/or both the N- and C- termini of the original protein and or deletions from the new N- and/or C-termini of the sequence rearranged proteins in the formulas shown above.

[0023] The flt3 receptor agonists of the present invention may contain amino acid substitutions, deletions and/or insertions.

[0024] A preferred embodiment of the present invention the linker (L) joining the N-terminus to the C-terminus is a polypeptide selected from the group consisting of:

GlyGlyGlySer SEQ ID NO:38;
GlyGlyGlySerGlyGlyGlySer SEQ ID NO:39;
GlyGlyGlySerGlyGlyGlySerGlyGlyGlySer SEQ ID NO:40;
SerGlyGlySerGlyGlySer SEQ ID NO:41;
GluPheGlyAsnMet SEQ ID NO:42;
GluPheGlyGlyAsnMet SEQ ID NO:43;
GluPheGlyGlyAsnGlyGlyAsnMet SEQ ID NO:44;
GlyGlySerAspMetAlaGly SEQ ID NO:45;
SerGlyGlyAsnGly SEQ ID NO:46;
SerGlyGlyAsnGlySerGlyGlyAsnGly SEQ ID NO:47;
SerGlyGlyAsnGlySerGlyGlyAsnGlySerGlyGlyAsnGly SEQ ID NO:48;
SerGlyGlySerGlySerGlyGlySerGly SEQ ID NO:49;
SerGlyGlySerGlySerGlyGlySerGlySerGlyGlySerGly SEQ ID NO:50;
GlyGlyGlySerGlyGly SEQ ID NO:51;
GlyGlyGlySerGlyGlyGly SEQ ID NO:52;
GlyGlyGlySerGlyGlyGlySerGlyGly SEQ ID NO:53;
GlyGlyGlySerGlyGlyGlySerGlyGlyGlySerGly SEQ ID NO:54;
GlyGlyGlySerGlyGlyGlySerGlyGlyGlySerGlyGlyGly SEQ ID NO:55;

GlyGlyGlySerGlyGlyGlySerGlyGlyGlySerGlyGlyGlySerGly GlyGlySerGly SEQ ID NO:56; GlyGlyGlySerGlyGlyGlySerGlyGlyGlySerGlyGlyGlySerGly GlyGlySerGlyGlyGlySerGlyGlyGlySerGly SEQ ID NO:148; ProProProTrpSerProArgProLeuGlyAlaThrAlaProThrAlaGly GlnProProLeu SEQ ID NO:149; ProProProTrpSerProArgProLeuGlyAlaThrAlaProThr SEQ ID NO:150; and ValGluThrValPheHisArgValSerGlnAspGlyLeuLeuThrSer SEQ ID NO:151.

[0025] The present invention also encompasses recombinant human flt3 receptor agonists co-administered or sequentially with one or more additional colony stimulating factors (CSF) including, cytokines, lymphokines, interleukins, hematopoietic growth factors which include but are not limited to GM-CSF, G-CSF, c-mpl ligand (also known as TPO or MGDF), M-CSF, erythropoietin (FLT3), IL-1, IL-4, IL-2, IL-3, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, LIF, human growth hormone, B-cell growth factor, B-cell differentiation factor, eosinophil differentiation factor and stem cell factor (SCF) also known as steel factor or c-kit ligand (herein collectively referred to as " factors"). These co-administered mixtures may be characterized by having the usual activity of both of the peptides or the mixture may be further characterized by having a biological or physiological activity greater than simply the additive function of the presence of the flt3 receptor agonists

or the second colony stimulating factor alone. The co-administration may also provide an enhanced effect on the activity or an activity different from that expected by the presence of the flt3 ligand or the second colony stimulating factor. The co-administration may also have an improved activity profile which may include reduction of undesirable biological activities associated with native human flt3 ligand. In addition to the list above, IL-3 variants taught in WO 94/12639 and WO 94/12638 fusion protein taught in WO 95/21197, and WO 95/21254 G-CSF receptor agonists disclosed in WO 97/12977, c-mpl receptor agonists disclosed in WO 97/12978, IL-3 receptor agonists disclosed in WO 97/12979 and multi-functional receptor agonists taught in WO 97/12985 can be co-administered with the polypeptides of the present invention. As used herein "IL-3 variants" refer to IL-3 variants taught in WO 94/12639 and WO 94/12638. As used herein "fusion proteins" refer to fusion protein taught in WO 95/21197, and WO 95/21254. As used herein "G-CSF receptor agonists" refer to G-CSF receptor agonists disclosed in WO 97/12978. As used herein "c-mpl receptor agonists" refer to c-mpl receptor agonists disclosed in WO 97/12978. As used herein "IL-3 receptor agonists" refer to IL-3 receptor agonists disclosed in WO 97/12979. As used herein

"multi-functional receptor agonists" refer to multi-functional receptor agonists taught in WO 97/12985.

[0026] In addition, it is envisioned that in vitro uses would include the ability to stimulate bone marrow and blood cell activation and growth before the expanded cells are infused into patients. Another intended use is for the production of dendritic cells both *in vivo* and *ex vivo*.

BRIEF DESCRIPTION OF THE FIGURES

[0027] Figure 1 schematically illustrates the sequence rearrangement of a protein. The N-terminus (N) and the C-terminus (C) of the native protein are joined through a linker, or joined directly. The protein is opened at a breakpoint creating a new N-terminus (new N) and a new C-terminus (new-C) resulting in a protein with a new linear amino acid sequence. A rearranged molecule may be synthesized *de novo* as linear molecule and not go through the steps of joining the original N-terminus and the C-terminus and opening of the protein at the breakpoint.

[0028] Figure 2 shows a schematic of Method I, for creating new proteins in which the original N-terminus and C-terminus of the native protein are joined with a linker and different N-terminus and C-terminus of the protein are created. In the example shown the sequence rearrangement results in a new gene encoding a protein with a new N-terminus created at amino acid 97

of the original protein, the original C-terminus (a.a. 174) joined to the amino acid 11 (a.a. 1- 10 are deleted) through a linker region and a new C-terminus created at amino acid 96 of the original sequence.

[0029] Figure 3 shows a schematic of Method II, for creating new proteins in which the original N-terminus and C-terminus of the native protein are joined without a linker and different N-terminus and C-terminus of the protein are created. In the example shown the sequence rearrangement results in a new gene encoding a protein with a new N-terminus created at amino acid 97 of the original protein, the original C-terminus (a.a. 174) joined to the original N-terminus and a new C-terminus created at amino acid 96 of the original sequence.

[0030] Figure 4 shows a schematic of Method III, for creating new proteins in which the original N-terminus and C-terminus of the native protein are joined with a linker and different N-terminus and C-terminus of the protein are created. In the example shown the sequence rearrangement results in a new gene encoding a protein with a new N-terminus created at amino acid 97 of the original protein, the original C-terminus (a.a. 174) joined to amino acid 1 through a linker region and a new C-terminus created at amino acid 96 of the original sequence.

[0031] Figure 5a and 5b shows the DNA sequence encoding the 209 amino acid mature form of flt3 ligand from Lyman et al. (*Oncogene* **11**:1165-1172, 1995).

[0032] Figure 6 shows the DNA sequence encoding the 134 amino acid soluble form of flt3 ligand from Lyman et al. (*Oncogene* **11**:1165-1172, 1995).

[0033] Figure 7 shows the bioactivity of the flt3 receptor agonists pMON32320 and pMON32321 compared to recombinant native flt3 (Genzyme) and pMON30237 (1-134 form of the flt3 ligand expressed by mammalian cell (BHK) culture) in the MUTZ-2 cell proliferation assay. MT = mock transfection.

DETAILED DESCRIPTION OF THE INVENTION

[0034] Flt3 receptor agonists of the present invention may be useful in the treatment of diseases characterized by decreased levels of hematopoietic cells.

[0035] A flt3 receptor agonist may be useful in the treatment or prevention of hematopoietic disorders. Many drugs may cause bone marrow suppression or hematopoietic deficiencies. Examples of such drugs are AZT, DDI, alkylating agents and anti-metabolites used in chemotherapy, antibiotics such as chloramphenicol, penicillin, gancyclovir, daunomycin and sulfa drugs, phenothiazones, tranquilizers such as meprobamate, analgesics such as aminopyrine and dipyrone, anti-convulsants such as phenytoin or carbamazepine, antithyroids such as propylthiouracil and methimazole and diuretics. flt3 receptor agonists may be useful in preventing or treating the bone marrow suppression or hematopoietic deficiencies which often occur in patients treated with these drugs.

[0036] Hematopoietic deficiencies may also occur as a result of viral, microbial or parasitic infections, burns and as a result of treatment for renal disease or renal failure, e.g., dialysis. The present peptide may be useful in treating such hematopoietic deficiency.

[0037] Another aspect of the present invention provides plasmid DNA vectors for use in the method of expression of these novel flt3 receptor agonists. These vectors contain the novel DNA sequences described above which code for the novel polypeptides of the invention. Appropriate vectors which can transform host cells capable of expressing the flt3 receptor agonists include expression vectors comprising nucleotide sequences coding for the flt3 receptor agonists joined to transcriptional and translational regulatory sequences which are selected according to the host cells used. Vectors incorporating modified sequences as described above are included in the present invention and are useful in the production of the modified flt3 receptor agonist polypeptides. The vector employed in the method also contains selected regulatory sequences in operative association with the DNA coding sequences of the invention and capable of directing the replication and expression thereof in selected host cells.

[0038] As another aspect of the present invention, there is provided a novel method for producing the novel family of human flt3 receptor agonists. The method of the present invention involves culturing suitable cells or cell line, which has been transformed with a vector containing a DNA sequence coding for expression of the novel flt3 receptor agonist polypeptide.

Suitable cells or cell lines may include various strains of bacteria such as *E. coli*, yeast, mammalian cells, or insect cells may be utilized as host cells in the method of the present invention.

[0039] Other aspects of the present invention are methods and therapeutic compositions for treating the conditions referred to above. Such compositions comprise a therapeutically effective amount of one or more of the flt3 receptor agonists of the present invention in a mixture with a pharmaceutically acceptable carrier. This composition can be administered either parenterally, intravenously or subcutaneously. When administered, the therapeutic composition for use in this invention is preferably in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such a parenterally acceptable protein solution, having due regard to pH, isotonicity, stability and the like, is within the skill of the art.

[0040] The dosage regimen involved in a method for treating the above-described conditions will be determined by the attending physician considering various factors which modify the action of drugs, e.g. the condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. Generally, a daily

regimen may be in the range of 0.5 - 150 μg/kg of non-glycosylated flt3 receptor agonists protein per kilogram of body weight. Dosages would be adjusted relative to the activity of a given receptor agonist and it would not be unreasonable to note that dosage regimens may include doses as low as 0.1 microgram and as high as 1 milligram per kilogram of body weight per day. In addition, there may exist specific circumstances where dosages of flt3 receptor agonist would be adjusted higher or lower than the range of 0.5 - 150 micrograms per kilogram of body weight. These include co-administration with other CSF or growth factors; co-administration with chemotherapeutic drugs and/or radiation; the use of glycosylated flt3 receptor agonists; and various patient-related issues mentioned earlier in this section. As indicated above, the therapeutic method and compositions may also include co-administration with other human factors. A non-exclusive list of other appropriate hematopoietins, CSFs and interleukins for simultaneous or serial co-administration with the polypeptides of the present invention includes GM-CSF, G-CSF, c-mpl ligand (also known as TPO or MGDF), M-CSF, erythropoietin (EPO), IL-1, IL-4, IL-2, IL-3, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, LIF, human growth hormone, B-cell growth factor, B-cell differentiation factor, eosinophil differentiation factor and stem cell factor (SCF) also known as

steel factor or c-kit ligand (herein collectively referred to as "factors"), or combinations thereof. In addition to the list above, IL-3 variants taught in WO 94/12639 and WO 94/12638 fusion protein taught in WO 95/21197, and WO 95/21254 G-CSF receptor agonists disclosed in WO 97/12977, c-mpl receptor agonists disclosed in WO 97/12978, IL-3 receptor agonists disclosed in WO 97/12979 and multi-functional receptor agonists taught in WO 97/12985 can be co-administered with the polypeptides of the present invention.

[0041] The flt3 receptor agonists of the present invention may be useful in the mobilization of hematopoietic progenitors and stem cells in peripheral blood. Peripheral blood derived progenitors have been shown to be effective in reconstituting patients in the setting of autologous marrow transplantation. Hematopoietic growth factors, including G-CSF and GM-CSF, have been shown to enhance the number of circulating progenitors and stem cells in the peripheral blood. This has simplified the procedure for peripheral stem cell collection and dramatically decreased the cost of the procedure by decreasing the number of pheresis required. The flt3 receptor agonist of the present invention may be useful in mobilization of stem cells and further enhance the efficacy of peripheral stem cell transplantation.

[0042] The flt3 receptor agonists of the present invention may also be useful in the ex vivo expansion of hematopoietic progenitors. Colony stimulating factors (CSFs), such as G-CSF, have been administered alone, co-administered with other CSFs, or in combination with bone marrow transplants subsequent to high dose chemotherapy to treat the neutropenia and which is often the result of such treatment. However the period of severe neutropenia may not be totally eliminated. The myeloid lineage, which is comprised of monocytes (macrophages), granulocytes (including neutrophils) and megakaryocytes, is critical in preventing infections and bleeding which can be life-threatening. Neutropenia may also be the result of disease, genetic disorders, drugs, toxins, radiation and many therapeutic treatments such as conventional oncology therapy.

[0043] Bone marrow transplants have been used to treat this patient population. However, several problems are associated with the use of bone marrow to reconstitute a compromised hematopoietic system including: 1) the number of stem cells in bone marrow or other tissues, such as spleen or peripheral blood, is limited, 2) Graft Versus Host Disease, 3) graft rejection and 4) possible contamination with tumor cells. Stem cells and progenitor cells make up a very small percentage of the nucleated cells in the bone marrow, spleen and peripheral blood. It is

clear that a dose response exists such that a greater number of multipotential hematopoietic progenitors will enhance hematopoietic recovery. Therefore, the in vitro expansion of stem cells should enhance hematopoietic recovery and patient survival. Bone marrow from an allogeneic donor has been used to provide bone marrow for transplant. However, Graft Versus Host Disease and graft rejection limit bone marrow transplantation even in recipients with HLA-matched sibling donors. An alternative to allogeneic bone marrow transplants is autologous bone marrow transplants. In autologous bone marrow transplants, some of the patient's own marrow is harvested prior to myeloablative therapy, e.g. high dose chemotherapy, and is transplanted back into the patient afterwards. Autologous transplants eliminate the risk of Graft Versus Host Disease and graft rejection. However, autologous bone marrow transplants still present problems in terms of the limited number of stems cells in the marrow and possible contamination with tumor cells. The limited number of multipotential hematopoietic progenitors may be overcome by ex-vivo expansion of the multipotential hematopoietic progenitors. In addition, stem cells can be specifically isolated based on the presence of specific surface antigens such as CD34+ in order to decrease tumor cell contamination of the marrow graft.

[0044] The following patents contain further details on separating stem cells, CD34+ cells, culturing the cells with hematopoietic factors, the use of the cells for the treatment of patients with hematopoietic disorders and the use of hematopoietic factors for cell expansion and gene therapy.

[0045] U.S. Pat. No. 5,061,620 relates to compositions comprising human hematopoietic stem cells provided by separating the stem cells from dedicated cells.

U.S. Pat. No. 5,199,942 describes a method for autologous hematopoietic cell transplantation comprising: (1) obtaining hematopoietic progenitor cells from a patient; (2) ex-vivo expansion of cells with a growth factor selected from the group consisting of IL-3, flt3 ligand, c-kit ligand, GM CSF, IL-1, GM-CSF/IL-3 fusion protein and combinations thereof; (3) administering cellular preparation to a patient.

U.S. Pat. No. 5,240,856 relates to a cell separator that includes an apparatus for automatically controlling the cell separation process.

WO 91/16116 describes devices and methods for selectively isolating and separating target cells from a mixture of cells.

WO 91/18972 describes methods for in vitro culturing of bone marrow, by incubating suspension of bone marrow cells, using a hollow fiber bioreactor.

WO 92/18615 relates to a process for maintaining and expanding bone marrow cells, in a culture medium containing specific mixtures of cytokines, for use in transplants.

WO 93/08268 describes a method for selectively expanding stem cells, comprising the steps of (a) separating CD34+ stem cells from other cells and (b) incubating the separated cells in a selective medium, such that the stem cells are selectively expanded.

WO 93/18136 describes a process for in vitro support of mammalian cells derived from peripheral blood.

WO 93/18648 relates to a composition comprising human neutrophil precursor cells with a high content of myeloblasts and promyelocytes for treating genetic or acquired neutropenia.

WO 94/08039 describes a method of enrichment for human hematopoietic stem cells by selection for cells which express c-kit protein.

WO 94/11493 describes a stem cell population that are CD34+ and small in size, which are isolated using a counterflow elutriation method.

WO 94/27698 relates to a method combining immunoaffinity separation and continuous flow centrifugal separation for the selective separation of a nucleated heterogeneous cell population from a heterogeneous cell mixture.

WO 94/25848 describes a cell separation apparatus for collection and manipulation of target cells.

The long term culturing of highly enriched CD34+ precursors of hematopoietic progenitor cells from human bone marrow in cultures containing IL-1α, IL-3, IL-6 or GM-CSF is discussed in Brandt et al (*J. Clin. Invest.* **86**:932-941, 1990).

[0046] One aspect of the present invention provides a method for selective ex-vivo expansion of stem cells. The term "stem cell" refers to the multipotential hematopoietic cells as well as early myeloid progenitor and precursors cells which can be isolated from bone marrow, spleen or peripheral blood. The term "expansion" refers to the proliferation and differentiation of the cells. The present invention provides a method for selective ex-vivo expansion of stem cells, comprising the steps of; (a) separating stem cells from other cells, (b) culturing the separated stem cells with a selective medium which contains a flt3 receptor agonist and optionally a second colony stimulating factor, and (c) harvesting the cultured stems cells. Stem cells, as well as committed progenitor cells destined to become neutrophils, erythrocytes, platelets, etc., may be distinguished from most other cells by the presence or absence of particular progenitor marker antigens, such as CD34, that are present on the surface of these cells and/or by morphological characteristics.

The phenotype for a highly enriched human stem cell fraction is reported as CD34+, Thy-1+ and lin-, but it is to be understood that the present invention is not limited to the expansion of this stem cell population. The CD34+ enriched human stem cell fraction can be separated by a number of reported methods, including affinity columns or beads, magnetic beads or flow cytometry using antibodies directed to surface antigens such as the CD34+. Further, physical separation methods such as counterflow elutriation may be used to enrich hematopoietic progenitors. The CD34+ progenitors are heterogeneous, and may be divided into several sub-populations characterized by the presence or absence of co-expression of different lineage associated cell surface associated molecules. The most immature progenitor cells do not express any known lineage associated markers, such as HLA-DR or CD38, but they may express CD90(thy-1). Other surface antigens such as CD33, CD38, CD41, CD71, HLA-DR or c-kit can also be used to selectively isolate hematopoietic progenitors. The separated cells can be incubated in selected medium in a culture flask, sterile bag or in hollow fibers. Various colony stimulating factors may be utilized in order to selectively expand cells. Representative factors that have been utilized for ex-vivo expansion of bone marrow include, c-kit ligand, IL-3, G-CSF, GM-CSF, IL-1, IL-6, IL-11, flt3 ligand or

combinations thereof. The proliferation of the stem cells can be monitored by enumerating the number of stem cells and other cells, by standard techniques (e.g. hemacytometer, CFU, LTCIC) or by flow cytometry prior and subsequent to incubation.

[0047] Several methods for ex-vivo expansion of stem cells have been reported utilizing a number of selection methods and expansion using various colony stimulating factors including c-kit ligand (Brandt et al., *Blood* **83**:1507-1514, 1994; McKenna et al., *Blood* **86**:3413-3420, 1995), IL-3 (Brandt et al., *Blood* **83**:1507-1514, 1994; Sato et al., *Blood* **82**:3600-3609, 1993), G-CSF (Sato et al., *Blood* **82**:3600-3609, 1993), GM-CSF (Sato et al., *Blood* **82**:3600-3609, 1993), IL-1 (Muench et al., *Blood* **81**:3463-3473, 1993), IL-6 (Sato et al., *Blood* **82**:3600-3609, 1993), IL-11 (Lemoli et al., *Exp. Hem.* **21**:1668-1672, 1993; Sato et al., *Blood* **82**:3600-3609, 1993), flt3 ligand (McKenna et al., *Blood* **86**:3413 3420, 1995) and/or combinations thereof (Brandt et al., *Blood* **83**:1507 1514, 1994; Haylock et al., *Blood* **80**:1405-1412, 1992, Koller et al., *Biotechnology* **11**:358-363, 1993; Lemoli et al., *Exp. Hem.* **21**:1668-1672, 1993), McKenna et al., *Blood* **86**:3413-3420, 1995; Muench et al., *Blood* **81**:3463-3473, 1993; Patchen et al., *Biotherapy* **7**:13-26, 1994; Sato et al., *Blood* **82**:3600-3609, 1993; Smith et al., *Exp. Hem.* **21**:870-877, 1993; Steen et al.,

Stem Cells **12**:214-224, 1994; Tsujino et al., *Exp. Hem.* **21**:1379-1386, 1993). Among the individual colony stimulating factors, hIL-3 has been shown to be one of the most potent in expanding peripheral blood CD34+ cells (Sato et al., *Blood* **82**:3600-3609, 1993; Kobayashi et al., *Blood* **73**:1836-1841, 1989). However, no single factor has been shown to be as effective as the combination of multiple factors. The present invention provides methods for ex vivo expansion that utilize novel flt3 receptor agonists.

[0048] Another aspect of the invention provides methods of sustaining and/or expanding hematopoietic precursor cells which includes inoculating the cells into a culture vessel which contains a culture medium that has been conditioned by exposure to a stromal cell line such as HS-5 (WO 96/02662, Roecklein and Torok-Strob, *Blood* **85**:997-1105, 1995) that has been supplemented with a flt3 receptor agonist of the present invention.

[0049] It is also envisioned that uses of flt3 receptor agonists of the present invention would include blood banking applications, where the flt3 receptor agonists are given to a patent to increase the number of blood cells and blood products are removed from the patient, prior to some medical procedure, and the blood products are stored and transfused back into the patient after the medical procedure. Additionally, it is

envisioned that uses of flt3 receptor agonists would include giving the flt3 receptor agonists to a blood donor prior to blood donation to increase the number of blood cells, thereby allowing the donor to safely give more blood.

[0050] Another projected clinical use of growth factors has been in the in vitro activation of hematopoietic progenitors and stem cells for gene therapy. Due to the long life-span of hematopoietic progenitor cells and the distribution of their daughter cells throughout the entire body, hematopoietic progenitor cells are good candidates for ex vivo gene transfection. In order to have the gene of interest incorporated into the genome of the hematopoietic progenitor or stem cell one needs to stimulate cell division and DNA replication. Hematopoietic stem cells cycle at a very low frequency which means that growth factors may be useful to promote gene transduction and thereby enhance the clinical prospects for gene therapy. Potential applications of gene therapy (review Crystal, *Science* **270**:404-410, 1995) include; 1) the treatment of many congenital metabolic disorders and immunodeficiencies (Kay and Woo, *Trends Genet.* **10**:253-257, 1994), 2) neurological disorders (Friedmann, *Trends Genet.* **10**:210-214, 1994), 3) cancer (Culver and Blaese, *Trends Genet.* **10**:174-178, 1994) and 4) infectious diseases (Gilboa and Smith, *Trends Genet.* **10**:139-144, 1994).

[0051] There are a variety of methods, known to those with skill in the art, for introducing genetic material into a host cell. A number of vectors, both viral and non-viral have been developed for transferring therapeutic genes into primary cells. Viral based vectors include; 1) replication deficient recombinant retrovirus (Boris-Lawrie and Temin, *Curr. Opin. Genet. Dev.* **3**:102-109, 1993; Boris-Lawrie and Temin, *Annal. New York Acad. Sci.* **716**:59-71, 1994; Miller, *Current Top. Microbiol. Immunol.* **158**:1-24, 1992) and replication-deficient recombinant adenovirus (Berkner, *BioTechniques* **6**:616-629, 1988; Berkner, *Current Top. Microbiol. Immunol.* **158**:39-66, 1992; Brody and Crystal, *Annal. New York Acad. Sci.* **716**:90-103, 1994). Non-viral based vectors include protein/DNA complexes (Cristiano et al., *PNAS USA.* **90**:2122-2126, 1993; Curiel et al., *PNAS USA* **88**:8850-8854, 1991; Curiel, *Annal. New York Acad. Sci.* **716**:36-58, 1994), electroporation and liposome mediated delivery such as cationic liposomes (Farhood et al., *Annal. New York Acad. Sci.* **716**:23-35, 1994).

[0052] The present invention provides an improvement to the existing methods of expanding hematopoietic cells, into which new genetic material has been introduced, in that it provides methods utilizing flt3 receptor agonists that may have improved biological activity and/or physical properties.

[0053] Another intended use of the flt-3 receptor agonists of the present invention is for the generation of larger numbers of dendritic cells, from precursors, to be used as adjuvants for immunization. Dendritic cells play a crucial role in the immune system. They are the professional antigen-presenting cells most efficient in the activation of resting T cells and are the major antigen-presenting cells for activation of naïve T cells *in vivo* and, thus, for initiation of primary immune responses. They efficiently internalize, process and present soluble tumor-specific antigens (Ag). Dendritic cells have the unique capacity to cluster naïve T cells and to respond to Ag encounter by rapid up-regulation of the expression of major histocompatability complex (MHC) and co-stimulatory molecules, the production of cytokines and migration towards lymphatic organs. Since dendritic cells are of central importance for sensitizing the host against a neoantigen for CD4-dependent immune responses, they may also play a crucial role in the generation and regulation of tumor immunity.

[0054] Dendritic cells originate from a bone marrow CD34+ precursor common to granulocytes and macrophages, and the existence of a separate dendritic cell colony-forming unit (CFU-DC) that give rise to pure dendritic cell colonies has been established in humans. In addition, a post-CFU CD14+

intermediate has been described with the potential to differentiate along the dendritic cell or the macrophage pathway under distinct cytokine conditions. This bipotential precursor is present in the bone marrow, cord blood and peripheral blood. Dendritic cells can be isolated by the cell specific marker, CD83, which is expressed on mature dendritic cells, to delineate the maturation of cultured dendritic cells.

[0055] Dendritic cells based strategies provide a method for enhancing immune response against tumors and infectious agents. AIDS is another disease for which dendritic cell based therapies can be used, since dendritic cells can play a major role in promoting HIV-1 replication. An immunotherapy requires the generation of dendritic cells from cancer patients, their *in vitro* exposure to tumor Ag, derived from surgically removed tumor masses, and reinjection of these cells into the tumor patients. Relatively crude membrane preparations of tumor cells will suffice as sources of tumor antigen, avoiding the necessity for molecular identification of the tumor antigen. The tumor antigen may also be synthetic peptides, carbohydrates, or nucleic acid sequences. In addition, concomitant administration of cytokines such as the flt-3 receptor agonists of the present invention may further facilitate the induction of tumor immunity. It is foreseen that the immunotherapy can be in an *in vivo* setting,

wherein the flt-3 receptor agonists of the present invention is administered to a patient, having a tumor, alone or with other hematopoietic growth factors to increase the number of dendritic cells and endogenous tumor antigen is presented on the dendritic cells. It is also envisioned that *in vivo* immunotherapy can be with exogenous antigen. It is also envisioned that the immunotherapy treatment may include the mobilization of dendritic cell precursors or mature dendritic, by administering the flt-3 receptor agonists of the present invention alone or with other hematopoietic growth factors to the patient, removing the dendritic cell precursors or mature dendritic cells from the patient, exposing the dendritic cells to antigen and returning the dendritic cells to the patient. Furthermore, the dendritic cells that have been removed can be cultured *ex vivo* with the flt-3 receptor agonists of the present invention alone or with other hematopoietic growth factors to increase the number of dendritic cells prior to exposure to antigen. Dendritic cells based strategies also provide a method for reducing the immune response in auto-immune diseases.

[0056] Studies on dendritic cells have been greatly hampered by difficulties in preparing the cells in sufficient numbers and in a reasonably pure form. In an ex-vivo cell expansion setting, granulocyte-macrophage colony-stimulating factor (GM-CSF) and

tumor necrosis factor-α (TNF-α) cooperate in the ex vivo generation of dendritic cells from hematopoietic progenitors (CD34+ cells) retrieved from bone marrow, cord blood, or peripheral blood and flk-2/flt-3 ligand and c-kit ligand (stem cell factor [SCF]) synergize to enhance the GM-CSF plus TNF-α induced generation of dendritic cells (Siena, S. *et al.* *Experimental Hematology* **23**:1463-1471, 1995). Also provide is a method of *ex vivo* expansion of dendritic cell precursors or mature dendritic cells using the flt-3 receptor agonists of the present invention to provide sufficient quantities of dendritic cells for immunotherapy.

Determination of the Linker

[0057] The length of the amino acid sequence of the linker can be selected empirically or with guidance from structural information, or by using a combination of the two approaches.

[0058] When no structural information is available, a small series of linkers can be prepared for testing using a design whose length is varied in order to span a range from 0 to 50 Å and whose sequence is chosen in order to be consistent with surface exposure (hydrophilicity, Hopp & Woods, *Mol. Immunol.* **20**: 483-489, 1983; Kyte & Doolittle, *J. Mol. Biol.* **157**:105-132, 1982; solvent exposed surface area, Lee & Richards, *J. Mol. Biol.* **55**:379-400, 1971) and the ability to adopt the necessary

conformation without deranging the configuration of the flt3 receptor agonist (conformationally flexible; Karplus & Schulz, *Naturwissenschaften* **72**:212-213, (1985). Assuming an average of translation of 2.0 to 3.8 Å per residue, this would mean the length to test would be between 0 to 30 residues, with 0 to 15 residues being the preferred range. Exemplary of such an empirical series would be to construct linkers using a cassette sequence such as Gly-Gly-Gly-Ser repeated n times, where n is 1, 2, 3 or 4. Those skilled in the art will recognize that there are many such sequences that vary in length or composition that can serve as linkers with the primary consideration being that they be neither excessively long nor short (cf., Sandhu, *Critical Rev. Biotech.* **12**: 437-462, 1992); if they are too long, entropy effects will likely destabilize the three-dimensional fold, and may also make folding kinetically impractical, and if they are too short, they will likely destabilize the molecule because of torsional or steric strain.

[0059] Those skilled in the analysis of protein structural information will recognize that using the distance between the chain ends, defined as the distance between the c-alpha carbons, can be used to define the length of the sequence to be used, or at least to limit the number of possibilities that must be tested in an empirical selection of linkers. They will also recognize

that it is sometimes the case that the positions of the ends of the polypeptide chain are ill-defined in structural models derived from x-ray diffraction or nuclear magnetic resonance spectroscopy data, and that when true, this situation will therefore need to be taken into account in order to properly estimate the length of the linker required. From those residues whose positions are well defined are selected two residues that are close in sequence to the chain ends, and the distance between their c-alpha carbons is used to calculate an approximate length for a linker between them. Using the calculated length as a guide, linkers with a range of number of residues (calculated using 2 to 3.8Å per residue) are then selected. These linkers may be composed of the original sequence, shortened or lengthened as necessary, and when lengthened the additional residues may be chosen to be flexible and hydrophilic as described above; or optionally the original sequence may be substituted for using a series of linkers, one example being the Gly-Gly-Gly-Ser (SEQ ID NO:38) cassette approach mentioned above; or optionally a combination of the original sequence and new sequence having the appropriate total length may be used.

Determination of the Amino and
Carboxyl Termini of flt3 Receptor Agonists

[0060] Sequences of flt3 receptor agonists capable of folding to biologically active states can be prepared by appropriate

selection of the beginning (amino terminus) and ending (carboxyl terminus) positions from within the original polypeptide chain while using the linker sequence as described above. Amino and carboxyl termini are selected from within a common stretch of sequence, referred to as a breakpoint region, using the guidelines described below. A novel amino acid sequence is thus generated by selecting amino and carboxyl termini from within the same breakpoint region. In many cases the selection of the new termini will be such that the original position of the carboxyl terminus immediately preceded that of the amino terminus. However, those skilled in the art will recognize that selections of termini anywhere within the region may function, and that these will effectively lead to either deletions or additions to the amino or carboxyl portions of the new sequence.

[0061] It is a central tenet of molecular biology that the primary amino acid sequence of a protein dictates folding to the three-dimensional structure necessary for expression of its biological function. Methods are known to those skilled in the art to obtain and interpret three-dimensional structural information using x-ray diffraction of single protein crystals or nuclear magnetic resonance spectroscopy of protein solutions. Examples of structural information that are relevant to the identification of breakpoint regions include the location and

type of protein secondary structure (alpha and 3-10 helices, parallel and anti-parallel beta sheets, chain reversals and turns, and loops; Kabsch & Sander, *Biopolymers* **22**: 2577-2637, 1983; the degree of solvent exposure of amino acid residues, the extent and type of interactions of residues with one another (Chothia, *Ann. Rev. Biochem.* **53**:537-572; 1984) and the static and dynamic distribution of conformations along the polypeptide chain (Alber & Mathews, *Methods Enzymol.* **154**: 511-533, 1987). In some cases additional information is known about solvent exposure of residues; one example is a site of post-translational attachment of carbohydrate which is necessarily on the surface of the protein. When experimental structural information is not available, or is not feasible to obtain, methods are also available to analyze the primary amino acid sequence in order to make predictions of protein tertiary and secondary structure, solvent accessibility and the occurrence of turns and loops. Biochemical methods are also sometimes applicable for empirically determining surface exposure when direct structural methods are not feasible; for example, using the identification of sites of chain scission following limited proteolysis in order to infer surface exposure (Gentile & Salvatore, *Eur. J. Biochem.* **218**:603-621, 1993). Thus using either the experimentally derived structural information or predictive methods (e.g., Srinivisan &

Rose *Proteins: Struct., Funct. & Genetics*, **22**: 81-99, 1995) the parental amino acid sequence is inspected to classify regions according to whether or not they are integral to the maintenance of secondary and tertiary structure. The occurrence of sequences within regions that are known to be involved in periodic secondary structure (alpha and 3-10 helices, parallel and anti-parallel beta sheets) are regions that should be avoided. Similarly, regions of amino acid sequence that are observed or predicted to have a low degree of solvent exposure are more likely to be part of the so-called hydrophobic core of the protein and should also be avoided for selection of amino and carboxyl termini. In contrast, those regions that are known or predicted to be in surface turns or loops, and especially those regions that are known not to be required for biological activity, are the preferred sites for location of the extremes of the polypeptide chain. Continuous stretches of amino acid sequence that are preferred based on the above criteria are referred to as a breakpoint region.

TABLE 1
OLIGONUCLEOTIDES

NCOFLT	CTGACCATGGCNACCCAGGACTGCTCCTTCCAA SEQ ID NO:57;
HIND160	ACTGAAGCTTAGGGCTGACACTGCAGCTCCAG SEQ ID NO:58;
HIND165	ACTGAAGCTTACAGGGTTGAGGAGTCGGGCTG SEQ ID NO:59;
FL23For	GACTGCCATGGCNACYCAGGAYTGYTCYTTYCAACACAGCCCCATC SEQ ID NO:60;
FH3AFor	GACTGCCATGGCNACYCAGGAYTGYTCYTTYCAACACAGCCCCATC SEQ ID NO:61;
SCF.REV	TGTCCAAACTCATCAATGTATC SEQ ID NO:62;
39FOR	CATGGCCATGGCCGACGAGGAGCTCTGCGGGGGCCTCT SEQ ID NO:63;
39REV	GCTAGAAGCTTACTGCAGGTTGGAGGCCACGGTGAC SEQ ID NO:64;
65FOR	CATGGCCATGGCCTCCAAGATGCAAGGCTTGCTGGAGC SEQ ID NO:65;
65REV	GCTAGAAGCTTACCCAGCGACAGTCTTGAGCCGCTC SEQ ID NO:66;
89FOR	CATGGCCATGGCCCCCCCCAGCTGTCTTCGCTTCGT SEQ ID NO:67;
89REV	GCTAGAAGCTTAGGGCTGAAAGGCACATTTGGTGACA SEQ ID NO:68;
L5A	CCCTGTCTGGCGGCAACGGCACCCAGGACTGCTCCTTCCAAC SEQ ID NO:69;
L10A	GCGGTAACGGCAGTGGAGGTAATGGCACCCAGGACTGCTCCTTCCAAC SEQ ID NO:70;
L15A	ACGGCAGTGGTGGCAATGGGAGCGGCGGAAATGGAACCCAGGACTGCTCCT TCCAAC SEQ ID NO:71;
L5B	GTGCCGTTGCCGCCAGACAGGGTTGAGGAGTCGGGCTG SEQ ID NO:72;
L10B	ATTACCTCCACTGCCGTTACCGCCTGACAGGGTTGAGGAGTCGGGCTG SEQ ID NO:73;
L15B	GCTCCCATTGCCACCACTGCCGTTACCTCCAGACAGGGTTGAGGA GTCGGGCTG SEQ ID NO:74;
L15C	GATGAGGATCCGGTGGCAATGGGAGCGGCGGAAATGGAACCCAGG ACTGCTCCTTCCACC SEQ ID NO:75;

L15D GATGACGGATCCGTTACCTCCAGACAGGGTTGAGGAGTCGGGCTG SEQ ID NO:76;

L15E GATGACGGATCCGGAGGTAATGGCACCCAGGACTGCTCCTTCCAAC SEQ ID NO:77;

339FOR2 GACTGCCATGGCCGACGAGGAGCTCTGCG SEQ ID NO:78;

339REV2 GACTCAAGCTTACTGCAGGTTGGAGGCC SEQ ID NO:79;

339-10FOR3 GACTCGGGATCCGGAGGTTCTGGCACCCAGGACTGCTCC SEQ ID NO:80;

339-15FOR2 GACTGGGATCCGGTGGCAGTGGGAGCGGCGGATCTGGAACC SEQ ID NO:81;

339REV3 GACTTGGGATCCACTACCTCCAGACAGGGTTGAGGAGTC SEQ ID NO:82;

FLN3 ACTGACGGATCCACCGCCCAGGGTTGAGGAGTCGGGCTG SEQ ID NO:83;

FLN7 ACTGACGGATCCACCTCCTGACCCACCGCCCAGGGTTGAGGAGTCGGGCTG SEQ ID NO:84;

FLN11 ACTGACGGATCCACCTCCTGACCCACCTCCTGACCCACCGCCCAG GGTTGAGGAGTCGGGCTG SEQ ID NO:85;

C-term ACGTAAAGCTTACAGGGTTGAGGAGTCG SEQ ID NO:86;

FLC3 GTCAGTGGATCCGGAGGTACCCAGGACTGCTCCTTCCAAC SEQ ID NO:87;

FLC4 GTCAGTGGATCCGGAGGTGGCACCCAGGACTGCTCCTTCCAAC SEQ ID NO:88;

FLC10 GTCAGTGGATCCGGAGGTGGCTCAGGGGGAGGTAGTGGTACCCAG GACTGCTCCTTCCAAC SEQ ID NO:89;

Flt36 GTTGCCATGGCNTCNAAYCTGCARGAYGARGARCTGTGCGGGGGCCTCTGG CGGCTG SEQ ID NO:90;

Flt37 GTTGCCATGGCNAAYCTGCARGAYGARGARCTGTGYGGGGGCCTCTGGCG GCTGGTC SEQ ID NO:91;

Flt38 GTTGCCATGGCNCTGCARGAYGARGARCTGTGYGGYGGCCTCTGGCGGCTG GTCCTG SEQ ID NO:92;

Flt39 GTTGCCATGGCNCARGAYGARGARCTGTGYGGYGGYCTCTGGCGGCTGGTC CTGGCA SEQ ID NO:93;

Flt40 GTTGCCATGGCNGAYGARGARCTGTGYGGYGGYCTCTGGCGGCTGGTCCTG GCACAG SEQ ID NO:94;

Flt41 GTTGCCATGGCNGARGARCTGTGYGGYGGYCTCTGGCGGCTGGTCCTGGCA

CAGCGC SEQ ID NO:95;

Flt42 GTTGCCATGGCNGARCTGTGYGGYGGYCTGTGGCGYCTGGTCCTGGCACAG CGCTGG SEQ ID NO:96;

Flt43 GTTGCCATGGCNCTGTGYGGYGGYCTGTGGCGYCTGGTCCTGGCACAGCGC TGGATG SEQ ID NO:97;

36REV TATGCAAGCTTAGGCCACGGTGACTGGGTA SEQ ID NO:98;

37REV TATGCAAGCTTAGGAGGCCACGGTGACTGG SEQ ID NO:99;

38REV TATGCAAGCTTAGTTGGAGGCCACGGTGAC SEQ ID NO:100;

39REV TATGCAAGCTTACAGGTTGGAGGCCACGGT SEQ ID NO:101;

40REV TATGCAAGCTTACTGCAGGTTGGAGGCCAC SEQ ID NO:102;

41REV TATGCAAGCTTAGTCCTGCAGGTTGGAGGC SEQ ID NO:103;

42REV TATGCAAGCTTACTCGTCCTGCAGGTTGGA SEQ ID NO:104;

43REV TATGCAAGCTTACTCCTCGTCCTGCAGGTT SEQ ID NO:105;_

TABLE 2
DNA SEQUENCES

pMON30237.seq

GCCACCCAGGACTGCTCCTTCCAACACAGCCCCATCTCCTCCGACTTCGC
TGTCAAAATCCGTGAGCTGTCTGACTACCTGCTTCAAGATTACCCAGTCA
CCGTGGCCTCCAACCTGCAGGACGAGGAGCTCTGCGGGGCGCTCTGGCGG
CTGGTCCTGGCACAGCGCTGGATGGAGCGGCTCAAGACTGTCGCTGGGTC
CAAGATGCAAGGCTTGCTGGAGCGCGTGAACACGGAGATACACTTTGTCA
CCAAATGTGCCTTTCAGCCCCCCCCCAGCTGTCTTCGCTTCGTCCAGACC
AACATCTCCCGCCTCCTGCAGGAGACCTCCGAGCAGCTGGTGGCGCTGAA
GCCCTGGATCACTCGCCAGAACTTCTCCCGGTGCCTGGAGCTGCAGTGTC
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pMON30238.seq

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pMON30239.seq

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pMON32329.seq

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pMON32330.seq

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pMON32342.seq

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pMON32320.seq

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SEQ ID NO:114;

pMON32322.seq

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SEQ ID NO:117;

pMON32325.seq

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FLT3N.seq

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FLT3C.seq

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pMON35713.seq

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pMON35714.seq

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CTT SEQ ID NO:142;

pMON 35718.seq

GCCACCAACATCTCCCGCCTCCTGCAGGAGACCTCCGAGCAGCTGGTGGCGCTGAAGCCCTGGATC
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GTCACCGTGGCCTCCAACCTGCAGGACGAGGAGCTCTGCGGGGGCCTCTGGCGGCTGGTCCTGGCA
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CAG SEQ ID NO:143;

TABLE 3
PROTEIN SEQUENCES

pMON30237.pep

AlaThrGlnAspCysSerPheGlnHisSerProIleSerSerAspPheAlaValLysIleArgGluLeuS
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aLeuTrpArgLeuValLeuAlaGlnArgTrpMetGluArgLeuLysThrValAlaGlySerLysMetGln
GlyLeuLeuGluArgValAsnThrGluIleHisPheValThrLysCysAlaPheGlnProProProSerC
ysLeuArgPheValGlnThrAsnIleSerArgLeuLeuGlnGluThrSerGluGlnLeuValAlaLeuLy
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pMON30238.pep

AlaThrGlnAspCysSerPheGlnHisSerProIleSerSerAspPheAlaValLysIleArgGluLeuS
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yLeuTrpArgLeuValLeuAlaGlnArgTrpMetGluArgLeuLysThrValAlaGlySerLysMetGln
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SEQ ID NO:2;

pMON30239.pep

AlaThrGlnAspCysSerPheGlnHisSerProIleSerSerAspPheAlaValLysIleArgGluLeuS
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pMON32329.pep

GlyThrGlnAspCysSerPheGlnHisSerProIleSerSerAspPheAlaValLysIleArgGluLeuS
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ysLeuArgPheValGlnThrAsnIleSerArgLeuLeuGlnGluThrSerGluGlnLeuValAlaLeuLy
sProTrpIleThrArgGlnAsnPheSerArgCysLeuGluLeuGlnCysGlnPro SEQ ID NO:4;

pMON32330.pep

GlyThrGlnAspCysSerPheGlnHisSerProIleSerSerAspPheAlaValLysIleArgGluLeuS
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pMON32341.pep

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GlyLeuLeuGluArgValAsnThrGluIleHisPheValThrLysCysAlaPheGlnProProProSerC
ysLeuArgPheValGlnThrAsnIleSerArgLeuLeuGlnGluThrSerGluGlnLeuValAlaLeuLy
sProTrpIleThrArgGlnAsnPheSerArgCysLeuGluLeuGlnCysGlnPro SEQ ID NO:6;

pMON32342.pep

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GlyLeuLeuGluArgValAsnThrGluIleHisPheValThrLysCysAlaPheGlnProProProSerC
ysLeuArgPheValGlnThrAsnIleSerArgLeuLeuGlnGluThrSerGluGlnLeuValAlaLeuLy
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SerGluGlnLeuValAlaLeuLysProTrpIleThrArgGlnAsnPheSerArgCysLeuGluLeuGlnC
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ysGlnProAspSerSerThrLeuSerGlyGlyAsnGlySerGlyGlyAsnGlyThrGlnAspCysSerPh
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TyrProValThrValAlaSerAsnLeuGln SEQ ID NO:9;

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SerGluGlnLeuValAlaLeuLysProTrpIleThrArgGlnAsnPheSerArgCysLeuGluLeuGlnC
ysGlnProAspSerSerThrLeuSerGlyGlyAsnGlyThrGlnAspCysSerPheGlnHisSerProIl
eSerSerAspPheAlaValLysIleArgGluLeuSerAspTyrLeuLeuGlnAspTyrProValThrVal
AlaSerAsnLeuGln SEQ ID NO:10;

pMON32323.pep

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AspSerSerThrLeuSerGlyGlyAsnGlySerGlyGlyAsnGlySerGlyGlyAsnGlyThrGlnAspC
ysSerPheGlnHisSerProIleSerSerAspPheAlaValLysIleArgGluLeuSerAspTyrLeuLe
uGlnAspTyrProValThrValAlaSerAsnLeuGlnAspGluGluLeuCysGlyGlyLeuTrpArgLeu
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AlaSerLysMetGlnGlyLeuLeuGluArgValAsnThrGluIleHisPheValThrLysCysAlaPheG
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nLeuValAlaLeuLysProTrpIleThrArgGlnAsnPheSerArgCysLeuGluLeuGlnCysGlnPro
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erProIleSerSerAspPheAlaValLysIleArgGluLeuSerAspTyrLeuLeuGlnAspTyrProVa
lThrValAlaSerAsnLeuGlnAspGluGluLeuCysGlyGlyLeuTrpArgLeuValLeuAlaGlnArg
TrpMetGluArgLeuLysThrValAlaGly SEQ ID NO:12;

pMON32325.pep

AlaSerLysMetGlnGlyLeuLeuGluArgValAsnThrGluIleHisPheValThrLysCysAlaPheG
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spPheAlaValLysIleArgGluLeuSerAspTyrLeuLeuGlnAspTyrProValThrValAlaSerAs
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LysThrValAlaGly SEQ ID NO:13;

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AlaProProSerCysLeuArgPheValGlnThrAsnIleSerArgLeuLeuGlnGluThrSerGluGlnL
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pSerSerThrLeuSerGlyGlyAsnGlySerGlyGlyAsnGlySerGlyGlyAsnGlyThrGlnAspCys
SerPheGlnHisSerProIleSerSerAspPheAlaValLysIleArgGluLeuSerAspTyrLeuLeuG
lnAspTyrProValThrValAlaSerAsnLeuGlnAspGluGluLeuCysGlyGlyLeuTrpArgLeuVa

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ValAsnThrGluIleHisPheValThrLysCysAlaPheGlnPro SEQ ID NO:14;

pMON32327.pep

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ProIleSerSerAspPheAlaValLysIleArgGluLeuSerAspTyrLeuLeuGlnAspTyrProValT
hrValAlaSerAsnLeuGlnAspGluGluLeuCysGlyGlyLeuTrpArgLeuValLeuAlaGlnArgTr
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HisPheValThrLysCysAlaPheGlnPro SEQ ID NO:15;

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pSerSerThrLeuSerGlyGlyAsnGlyThrGlnAspCysSerPheGlnHisSerProIleSerSerAsp
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CysAlaPheGlnPro SEQ ID NO:16;

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SerGluGlnLeuValAlaLeuLysProTrpIleThrArgGlnAsnPheSerArgCysLeuGluLeuGlnC
ysGlnProAspSerSerThrLeuSerGlyGlySerGlySerGlyGlySerGlySerGlyGlySerGlyTh
rGlnAspCysSerPheGlnHisSerProIleSerSerAspPheAlaValLysIleArgGluLeuSerAsp
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pMON32350.pep

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SerGluGlnLeuValAlaLeuLysProTrpIleThrArgGlnAsnPheSerArgCysLeuGluLeuGlnC
ysGlnProAspSerSerThrLeuSerGlyGlySerGlySerGlyGlySerGlyThrGlnAspCysSerPh
eGlnHisSerProIleSerSerAspPheAlaValLysIleArgGluLeuSerAspTyrLeuLeuGlnAsp
TyrProValThrValAlaSerAsnLeuGln SEQ ID NO:18;

FLT3N.pep

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yGlyLeuTrpArgLeuValLeuAlaGlnArgTrpMetGluArgLeuLysThrValAlaGlySerLysMet
GlnGlyLeuLeuGluArgValAsnThrGluIleHisPheValThrLysCysAlaPheGlnProProProS
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LeuGlyGlyGlySer SEQ ID NO:19;

FLT3C.pep

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LysMetGlnGlyLeuLeuGluArgValAsnThrGluIleHisPheValThrLysCysAlaPheGlnProP
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SerThrLeu SEQ ID NO:20;

FLT7N.pep

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GlnGlyLeuLeuGluArgValAsnThrGluIleHisPheValThrLysCysAlaPheGlnProProProS
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SerSerThrLeu SEQ ID NO:22;

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GlnGlyLeuLeuGluArgValAsnThrGluIleHisPheValThrLysCysAlaPheGlnProProProS

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LeuGlyGlyGlySerGlyGlyGlySerGlyGlyGlySer SEQ ID NO:23;

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SerGluGlnLeuValAlaLeuLysProTrpIleThrArgGlnAsnPheSerArgCysLeuGluLeuGlnC
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ValAlaSerAsnLeuGln SEQ ID NO:25;

pMON32366.pep

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SerGluGlnLeuValAlaLeuLysProTrpIleThrArgGlnAsnPheSerArgCysLeuGluLeuGlnC
ysGlnProAspSerSerThrLeuGlyGlyGlySerGlyGlyGlyThrGlnAspCysSerPheGlnHisSe
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ThrValAlaSerAsnLeuGln SEQ ID NO:26;

pMON32367.pep

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pMON32368.pep

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TyrLeuLeuGlnAspTyrProValThrValAlaSerAsnLeuGln SEQ ID NO:29;

pMON32370.pep

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NO:30;

pMON35712.pep

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pMON35713.pep

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sPheValThrLysCysAlaPheGlnProProProSerCysLeuArgPheValGlnThrAsnIleSerArg
LeuLeuGlnGluThrSerGluGlnLeuValAlaLeuLysProTrpIleThrArgGlnAsnPheSerArgC

ysLeuGluLeuGlnCysGlnProAspSerSerThrLeuGlyGlyGlySerGlyGlyGlySerGlyGlyGl
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pMON35714.pep

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rSerGluGlnLeuValAlaLeuLysProTrpIleThrArgGlnAsnPheSerArgCysLeuGluLeuGln
CysGlnProAspSerSerThrLeuGlyGlyGlySerGlyGlyGlySerGlyGlyGlySerGlyGlyGlyT
hrGlnAspCysSerPheGlnHisSerProIleSerSerAspPheAlaValLysIleArgGluLeuSerAs
pTyrLeuLeuGlnAspTyrProValThrValAlaSerAsnLeuGlnAspGluGluLeuCysGlyGlyLeu
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pMON35715.pep

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nLeuValAlaLeuLysProTrpIleThrArgGlnAsnPheSerArgCysLeuGluLeuGlnCysGlnPro
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ysSerPheGlnHisSerProIleSerSerAspPheAlaValLysIleArgGluLeuSerAspTyrLeuLe
uGlnAspTyrProValThrValAlaSerAsnLeuGlnAspGluGluLeuCysGlyGlyLeuTrpArgLeu
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pSerSerThrLeuGlyGlyGlySerGlyGlyGlySerGlyGlyGlySerGlyGlyGlyThrGlnAspCys
SerPheGlnHisSerProIleSerSerAspPheAlaValLysIleArgGluLeuSerAspTyrLeuLeuG
lnAspTyrProValThrValAlaSerAsnLeuGlnAspGluGluLeuCysGlyGlyLeuTrpArgLeuVa
lLeuAlaGlnArgTrpMetGluArgLeuLysThrValAlaGlySerLysMetGlnGlyLeuLeuGluArg
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pMON35717.pep

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yGlyGlySerGlyGlyGlySerGlyGlyGlySerGlyGlyGlyThrGlnAspCysSerPheGlnHisSer
ProIleSerSerAspPheAlaValLysIleArgGluLeuSerAspTyrLeuLeuGlnAspTyrProValT
hrValAlaSerAsnLeuGlnAspGluGluLeuCysGlyGlyLeuTrpArgLeuValLeuAlaGlnArgTr
pMetGluArgLeuLysThrValAlaGlySerLysMetGlnGlyLeuLeuGluArgValAsnThrGluIle
HisPheValThrLysCysAlaPheGlnProProProSerCysLeu SEQ ID NO:36;

pMON35718.pep

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yGlyGlySerGlyGlyGlySerGlyGlyGlyThrGlnAspCysSerPheGlnHisSerProIleSerSer

AspPheAlaValLysIleArgGluLeuSerAspTyrLeuLeuGlnAspTyrProValThrValAlaSerA
snLeuGlnAspGluGluLeuCysGlyGlyLeuTrpArgLeuValLeuAlaGlnArgTrpMetGluArgLe
uLysThrValAlaGlySerLysMetGlnGlyLeuLeuGluArgValAsnThrGluIleHisPheValThr
LysCysAlaPheGlnProProProSerCysLeuArgPheValGln SEQ ID NO:37;

Materials and Methods

Recombinant DNA methods

[0062] Unless noted otherwise, all specialty chemicals were obtained from Sigma Co., (St. Louis, MO). Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA) or Boehringer Mannheim (Indianapolis, IN).

Transformation of *E. coli* strains

[0063] *E. coli* strains, such as DH5α™ (Life Technologies, Gaithersburg, MD) and TG1 (Amersham Corp., Arlington Heights, IL) are used for transformation of ligation reactions and are the source of plasmid DNA for transfecting mammalian cells. *E. coli* strains, such as MON105 and JM101, can be used for expressing the flt3 receptor agonist of the present invention in the cytoplasm or periplasmic space.

MON105 ATCC#55204: F-, lamda-,IN(rrnD, rrE)1, rpoD+, rpoH358

DH5α™: F-, phi80dlacZdeltaM15, delta(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-,mk+), phoA, supE44lamda-, thi-1, gyrA96, relA1

TG1: delta(lac-pro), supE, thi-1, hsdD5/F'(traD36, proA+B+, lacIq, lacZdeltaM15)

[0064] DH5α™ Subcloning efficiency cells are purchased as competent cells and are ready for transformation using the

manufacturer's protocol, while both *E. coli* strains TG1 and MON105 are rendered competent to take up DNA using a $CaCl_2$ method. Typically, 20 to 50 mL of cells are grown in LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 150 mM NaCl) to a density of approximately 1.0 optical density unit at 600 nanometers (OD600) as measured by a Baush & Lomb Spectronic spectrophotometer (Rochester, NY). The cells are collected by centrifugation and resuspended in one-fifth culture volume of $CaCl_2$ solution (50 mM $CaCl_2$, 10 mM Tris-Cl, pH 7.4) and are held at 4°C for 30 minutes. The cells are again collected by centrifugation and resuspended in one-tenth culture volume of $CaCl_2$ solution. Ligated DNA is added to 0.2mL of these cells, and the samples are held at 4°C for 1 hour. The samples are shifted to 42°C for two minutes and 1mL of LB is added prior to shaking the samples at 37°C for one hour. Cells from these samples are spread on plates (LB medium plus 1.5% Bacto-agar) containing either ampicillin (100 micrograms/mL, ug/mL) when selecting for ampicillin-resistant transformants, or spectinomycin (75 ug/mL) when selecting for spectinomycin-resistant transformants. The plates are incubated overnight at 37°C. Single colonies are picked, grown in LB supplemented with

appropriate antibiotic for 6-16 hours at 37°C with shaking. Colonies are picked and inoculated into LB plus appropriate antibiotic (100 ug/mL ampicillin or 75 ug/mL spectinomycin) and are grown at 37°C while shaking. Before harvesting the cultures, 1 ul of cells are analyzed by PCR for the presence of a flt3 receptor agonist gene. The PCR is carried out using a combination of primers that anneal to the flt3 receptor agonist gene and/or vector. After the PCR is complete, loading dye is added to the sample followed by electrophoresis as described earlier. A gene has been ligated to the vector when a PCR product of the expected size is observed.

Methods for creation of genes
with new N-terminus/C-terminus

Method I. Creation of genes with new N-terminus/C-terminus which contain a linker region.

[0065] Genes with new N-terminus/C-terminus which contain a linker region separating the original C-terminus and N-terminus can be made essentially following the method described in L. S. Mullins, et al *J. Am. Chem. Soc.* **116**, 5529-5533 (1994). Multiple steps of polymerase chain reaction (PCR) amplifications are used to rearrange the DNA sequence encoding the primary amino acid sequence of the protein. The steps are illustrated in Figure 2.

[0066] In the first step, the primer set ("new start" and "linker start") is used to create and amplify, from the original gene sequence, the DNA fragment ("Fragment Start") that contains the sequence encoding the new N-terminal portion of the new protein followed by the linker that connects the C-terminal and N-terminal ends of the original protein. In the second step, the primer set ("new stop" and "linker stop") is used to create and amplify, from the original gene sequence, the DNA fragment ("Fragment Stop") that encodes the same linker as used above, followed by the new C-terminal portion of the new protein. The "new start" and "new stop" primers are designed to include the appropriate restriction enzyme recognition sites which allow cloning of the new gene into expression plasmids. Typical PCR conditions are one cycle 95°C melting for two minutes; 25 cycles 94°C denaturation for one minute, 50°C annealing for one minute and 72°C extension for one minute; plus one cycle 72°C extension for seven minutes. A Perkin Elmer GeneAmp PCR Core Reagents kit is used. A 100 ul reaction contains 100 pmole of each primer and one ug of template DNA; and 1x PCR buffer, 200 uM dGTP, 200 uM dATP, 200 uM dTTP, 200 uM dCTP, 2.5 units AmpliTaq DNA polymerase and 2 mM $MgCl_2$. PCR reactions are performed in a Model 480 DNA thermal cycler (Perkin Elmer Corporation, Norwalk, CT).

[0067] "Fragment Start" and "Fragment Stop", which have complementary sequence in the linker region and the coding sequence for the two amino acids on both sides of the linker, are joined together in a third PCR step to make the full-length gene encoding the new protein. The DNA fragments "Fragment Start" and "Fragment Stop" are resolved on a 1% TAE gel, stained with ethidium bromide and isolated using a Qiaex Gel Extraction kit (Qiagen). These fragments are combined in equimolar quantities, heated at 70°C for ten minutes and slow cooled to allow annealing through their shared sequence in "linker start" and "linker stop". In the third PCR step, primers "new start" and "new stop" are added to the annealed fragments to create and amplify the full-length new N-terminus/C-terminus gene. Typical PCR conditions are one cycle 95°C melting for two minutes; 25 cycles 94°C denaturation for one minute, 60°C annealing for one minute and 72°C extension for one minute; plus one cycle 72°C extension for seven minutes. A Perkin Elmer GeneAmp PCR Core Reagents kit is used. A 100 ul reaction contains 100 pmole of each primer and approximately 0.5 ug of DNA; and 1x PCR buffer, 200 uM dGTP, 200 uM dATP, 200 uM dTTP, 200 uM dCTP, 2.5 units AmpliTaq DNA polymerase and 2 mM $MgCl_2$. PCR reactions are purified using a Wizard PCR Preps kit (Promega).

Method II. Creation of genes with new N-terminus/C-terminus without a linker region.

[011] New N-terminus/C-terminus genes without a linker joining the original N-terminus and C-terminus can be made using two steps of PCR amplification and a blunt end ligation. The steps are illustrated in Figure 3. In the first step, the primer set ("new start" and "P-bl start") is used to create and amplify, from the original gene sequence, the DNA fragment ("Fragment Start") that contains the sequence encoding the new N-terminal portion of the new protein. In the second step, the primer set ("new stop" and "P-bl stop") is used to create and amplify, from the original gene sequence, the DNA fragment ("Fragment Stop") that contains the sequence encoding the new C-terminal portion of the new protein. The "new start" and "new stop" primers are designed to include appropriate restriction sites which allow cloning of the new gene into expression vectors. Typical PCR conditions are one cycle 95°C melting for two minutes; 25 cycles 94°C denaturation for one minute, 50°C annealing for 45 seconds and 72°C extension for 45 seconds. Deep Vent polymerase (New England Biolabs) is used to reduce the occurrence of overhangs in conditions recommended by the manufacturer. The "P-bl start" and "P-bl stop" primers are phosphorylated at the end to aid in the subsequent blunt end ligation of "Fragment Start" and "Fragment

Stop" to each other. A 100 ul reaction contained 150 pmole of each primer and one ug of template DNA; and 1x Vent buffer (New England Biolabs), 300 uM dGTP, 300 uM dATP, 300 uM dTTP, 300 uM dCTP, and 1 unit Deep Vent polymerase. PCR reactions are performed in a Model 480 DNA thermal cycler (Perkin Elmer Corporation, Norwalk, CT). PCR reaction products are purified using a Wizard PCR Preps kit (Promega).

[0068] The primers are designed to include appropriate restriction enzyme recognition sites which allow for the cloning of the new gene into expression vectors. Typically "Fragment Start" is designed to create a NcoI restriction site , and "Fragment Stop" is designed to create a HindIII restriction site. Restriction digest reactions are purified using a Magic DNA Clean-up System kit (Promega). Fragments Start and Stop are resolved on a 1% TAE gel, stained with ethidium bromide and isolated using a Qiaex Gel Extraction kit (Qiagen). These fragments are combined with and annealed to the ends of the ~ 3800 base pair NcoI/HindIII vector fragment of pMON3934 by heating at 50°C for ten minutes and allowed to slow cool. The three fragments are ligated together using T4 DNA ligase (Boehringer Mannheim). The result is a plasmid containing the full-length new N-terminus/C-terminus gene. A portion of the ligation reaction is used to transform *E. coli* strain DH5α cells

(Life Technologies, Gaithersburg, MD). Plasmid DNA is purified and sequence confirmed as below.

Method III. Creation of new N-terminus/C-terminus genes by tandem-duplication method

[0069] New N-terminus/C-terminus genes can be made based on the method described in R. A. Horlick, et al *Protein Eng.* **5**:427-431 (1992). Polymerase chain reaction (PCR) amplification of the new N-terminus/C-terminus genes is performed using a tandemly duplicated template DNA. The steps are illustrated in Figure 4.

[0070] The tandemly-duplicated template DNA is created by cloning and contains two copies of the gene separated by DNA sequence encoding a linker connecting the original C- and N-terminal ends of the two copies of the gene. Specific primer sets are used to create and amplify a full-length new N terminus/C-terminus gene from the tandemly-duplicated template DNA. These primers are designed to include appropriate restriction sites which allow for the cloning of the new gene into expression vectors. Typical PCR conditions are one cycle 95°C melting for two minutes; 25 cycles 94°C denaturation for one minute, 50°C annealing for one minute and 72°C extension for one minute; plus one cycle 72°C extension for seven minutes. A Perkin Elmer GeneAmp PCR Core Reagents kit (Perkin Elmer

Corporation, Norwalk, CT) is used. A 100 ul reaction contains 100 pmole of each primer and one ug of template DNA; and 1x PCR buffer, 200 uM dGTP, 200 uM dATP, 200 uM dTTP, 200 uM dCTP, 2.5 units AmpliTaq DNA polymerase and 2 mM $MgCl_2$. PCR reactions are performed in a Model 480 DNA thermal cycler (Perkin Elmer Corporation, Norwalk, CT). PCR reactions are purified using a Wizard PCR Preps kit (Promega).

DNA isolation and characterization

[0071] Plasmid DNA can be isolated by a number of different methods and using commercially available kits known to those skilled in the art. A few such methods are shown herein. Plasmid DNA is isolated using the Promega Wizard™ Miniprep kit (Madison, WI), the Qiagen QIAwell Plasmid isolation kits (Chatsworth, CA) or Qiagen Plasmid Midi kit. These kits follow the same general procedure for plasmid DNA isolation. Briefly, cells are pelleted by centrifugation (5000 x g), plasmid DNA released with sequential NaOH/acid treatment, and cellular debris is removed by centrifugation (10000 x g). The supernatant (containing the plasmid DNA) is loaded onto a column containing a DNA-binding resin, the column is washed, and plasmid DNA eluted with TE. After screening for the colonies with the plasmid of interest, the *E. coli* cells are inoculated into 50-100 mLs of LB plus appropriate antibiotic for overnight growth at 37°C in an air

incubator while shaking. The purified plasmid DNA is used for DNA sequencing, further restriction enzyme digestion, additional subcloning of DNA fragments and transfection into mammalian, *E. coli* or other cells.

Sequence confirmation

[0072] Purified plasmid DNA is resuspended in dH_2O and quantitated by measuring the absorbance at 260/280 nm in a Bausch and Lomb Spectronic 601 UV spectrometer. DNA samples are sequenced using ABI PRISM™ DyeDeoxy™ terminator sequencing chemistry (Applied Biosystems Division of Perkin Elmer Corporation, Lincoln City, CA) kits (Part Number 401388 or 402078) according to the manufacturers suggested protocol usually modified by the addition of 5% DMSO to the sequencing mixture. Sequencing reactions are performed in a Model 480 DNA thermal cycler (Perkin Elmer Corporation, Norwalk, CT) following the recommended amplification conditions. Samples are purified to remove excess dye terminators with Centri-Sep™ spin columns (Princeton Separations, Adelphia, NJ) and lyophilized. Fluorescent dye labeled sequencing reactions are resuspended in deionized formamide, and sequenced on denaturing 4.75% polyacrylamide-8M urea gels using an ABI Model 373A automated DNA sequencer. Overlapping DNA sequence fragments are analyzed and

assembled into master DNA contigs using Sequencher DNA analysis software (Gene Codes Corporation, Ann Arbor, MI).

Expression of flt3 receptor agonists in mammalian cells

Mammalian Cell Transfection/Production of Conditioned Media

[0073] The BHK-21 cell line can be obtained from the ATCC (Rockville, MD). The cells are cultured in Dulbecco's modified Eagle media (DMEM/high-glucose), supplemented to 2mM (mM) L-glutamine and 10% fetal bovine serum (FBS). This formulation is designated BHK growth media. Selective media is BHK growth media supplemented with 453 units/mL hygromycin B (Calbiochem, San Diego, CA). The BHK-21 cell line was previously stably transfected with the HSV transactivating protein VP16, which transactivates the IE110 promoter found on the plasmid pMON3359 (See Hippenmeyer et al., *Bio/Technology*, pp.1037-1041, 1993). The VP16 protein drives expression of genes inserted behind the IE110 promoter. BHK-21 cells expressing the transactivating protein VP16 are designated BHK-VP16. The plasmid pMON1118 (See Highkin et al., *Poultry Sci.*, **70**: 970-981, 1991) expresses the hygromycin resistance gene from the SV40 promoter. A similar plasmid is available from ATCC, pSV2-hph.

[0074] BHK-VP16 cells are seeded into a 60 millimeter (mm) tissue culture dish at 3 X 10^5 cells per dish 24 hours prior to transfection. Cells are transfected for 16 hours in 3 mL of

"OPTIMEM"™ (Gibco-BRL, Gaithersburg, MD) containing 10 ug of plasmid DNA containing the gene of interest, 3 ug hygromycin resistance plasmid, pMON1118, and 80 ug of Gibco-BRL "LIPOFECTAMINE"™ per dish. The media is subsequently aspirated and replaced with 3 mL of growth media. At 48 hours post-transfection, media from each dish is collected and assayed for activity (transient conditioned media). The cells are removed from the dish by trypsin-EDTA, diluted 1:10 and transferred to 100 mm tissue culture dishes containing 10 mL of selective media. After approximately 7 days in selective media, resistant cells grow into colonies several millimeters in diameter. The colonies are removed from the dish with filter paper (cut to approximately the same size as the colonies and soaked in trypsin/EDTA) and transferred to individual wells of a 24 well plate containing 1 mL of selective media. After the clones are grown to confluence, the conditioned media is re-assayed, and positive clones are expanded into growth media.

Expression of flt3 receptor agonists in *E. coli*

[0075] *E. coli* strain MON105 or JM101 harboring the plasmid of interest are grown at 37°C in M9 plus casamino acids medium with shaking in a air incubator Model G25 from New Brunswick Scientific (Edison, New Jersey). Growth is monitored at OD600 until it reaches a value of 1, at which time nalidixic acid (10

milligrams/mL) in 0.1 N NaOH is added to a final concentration of 50 µg/mL. The cultures are then shaken at 37°C for three to four additional hours. A high degree of aeration is maintained throughout culture period in order to achieve maximal production of the desired gene product. The cells are examined under a light microscope for the presence of inclusion bodies (IB). One mL aliquots of the culture are removed for analysis of protein content by boiling the pelleted cells, treating them with reducing buffer and electrophoresis via SDS-PAGE (see Maniatis et al. Molecular Cloning: A Laboratory Manual, 1982). The culture is centrifuged (5000 x g) to pellet the cells.

[0076] Additional strategies for achieving high-level expression of genes in E. coli can be found in Savvas, C.M. (*Microbiological Reviews* **60**;512-538, 1996).

Inclusion Body preparation, Extraction, Refolding, Dialysis, DEAE Chromatography, and Characterization of the flt3 receptor agonists which accumulate as inclusion bodies in *E. coli*.

Isolation of Inclusion Bodies:

[0077] The cell pellet from a 330 mL *E. coli* culture is resuspended in 15 mL of sonication buffer (10 mM 2-amino-2-(hydroxymethyl) 1,3-propanediol hydrochloride (Tris-HCl), pH 8.0 + 1 mM ethylenediaminetetraacetic acid (EDTA)). These resuspended cells are sonicated using the microtip probe of a

Sonicator Cell Disruptor (Model W-375, Heat Systems-Ultrasonics, Inc., Farmingdale, New York). Three rounds of sonication in sonication buffer followed by centrifugation are employed to disrupt the cells and wash the inclusion bodies (IB). The first round of sonication is a 3 minute burst followed by a 1 minute burst, and the final two rounds of sonication are for 1 minute each.

Extraction and refolding of proteins from inclusion body pellets:

[0078] Following the final centrifugation step, the IB pellet is resuspended in 10 mL of 50 mM Tris-HCl, pH 9.5, 8 M urea and 5 mM dithiothreitol (DTT) and stirred at room temperature for approximately 45 minutes to allow for denaturation of the expressed protein.

[0079] The extraction solution is transferred to a beaker containing 70 mL of 5mM Tris-HCl, pH 9.5 and 2.3 M urea and gently stirred while exposed to air at 4°C for 18 to 48 hours to allow the proteins to refold. Refolding is monitored by analysis on a Vydac (Hesperia, Ca.) C18 reversed phase high pressure liquid chromatography (RP-HPLC) column (0.46x25 cm). A linear gradient of 40% to 65% acetonitrile, containing 0.1% trifluoroacetic acid (TFA), is employed to monitor the refold. This gradient is developed over 30 minutes at a flow rate of 1.5

mL per minute. Denatured proteins generally elute later in the gradient than the refolded proteins.

Purification:

[0080] Following the refold, contaminating *E. coli* proteins are removed by acid precipitation. The pH of the refold solution is titrated to between pH 5.0 and pH 5.2 using 15% (v/v) acetic acid (HOAc). This solution is stirred at 4°C for 2 hours and then centrifuged for 20 minutes at 12,000 x g to pellet any insoluble protein.

[0081] The supernatant from the acid precipitation step is dialyzed using a Spectra/Por 3 membrane with a molecular weight cut off (MWCO) of 3,500 daltons. The dialysis is against 2 changes of 4 liters (a 50-fold excess) of 10mM Tris-HCl, pH 8.0 for a total of 18 hours. Dialysis lowers the sample conductivity and removes urea prior to DEAE chromatography. The sample is then centrifuged (20 minutes at 12,000 x g) to pellet any insoluble protein following dialysis.

[0082] A Bio-Rad Bio-Scale DEAE2 column (7 x 52 mm) is used for ion exchange chromatography. The column is equilibrated in a buffer containing 10mM Tris-HCl, pH 8.0. The protein is eluted using a 0-to-500 mM sodium chloride (NaCl) gradient, in equilibration buffer, over 45 column volumes. A flow rate of 1 mL per minute is used throughout the run. Column fractions (2 mL

per fraction) are collected across the gradient and analyzed by RP HPLC on a Vydac (Hesperia, Ca.) C18 column (0.46 x 25 cm). A linear gradient of 40% to 65% acetonitrile, containing 0.1% trifluoroacetic acid (TFA), is employed. This gradient is developed over 30 minutes at a flow rate of 1.5 mL per minute. Pooled fractions are then dialyzed against 2 changes of 4 liters (50-to-500-fold excess) of 10 mM ammonium acetate (NH_4Ac), pH 4.0 for a total of 18 hours. Dialysis is performed using a Spectra/Por 3 membrane with a MWCO of 3,500 daltons. Finally, the sample is sterile filtered using a 0.22µm syringe filter (µStar LB syringe filter, Costar, Cambridge, Ma.), and stored at 4°C.

[0083] In some cases the folded proteins can be affinity purified using affinity reagents such as mAbs or receptor subunits attached to a suitable matrix. Alternatively, (or in addition) purification can be accomplished using any of a variety of chromatographic methods such as: ion exchange, gel filtration or hydrophobic chromatography or reversed phase HPLC.

[0084] These and other protein purification methods are described in detail in Methods in Enzymology, Volume 182 'Guide to Protein Purification' edited by Murray Deutscher, Academic Press, San Diego, CA (1990).

Protein Characterization:

[0085] The purified protein is analyzed by RP-HPLC, electrospray mass spectrometry, and SDS-PAGE. The protein quantitation is done by amino acid composition, RP-HPLC, and Bradford protein determination. In some cases tryptic peptide mapping is performed in conjunction with electrospray mass spectrometry to confirm the identity of the protein.

Methylcellulose Assay

[0086] This assay reflects the ability of colony stimulating factors to stimulate normal bone marrow cells to produce different types of hematopoietic colonies *in vitro* (Bradley et al., *Aust. Exp Biol. Sci.* **44**:287-300, **1**966), Pluznik et al., *J. Cell Comp. Physio* **66**:319-324, 1965).

Methods

[0087] Approximately 30 mL of fresh, normal, healthy bone marrow aspirate are obtained from individuals following informed consent. Under sterile conditions samples are diluted 1:5 with a 1X PBS (#14040.059 Life Technologies, Gaithersburg, MD.) solution in a 50 mL conical tube (#25339-50 Corning, Corning MD). Ficoll (Histopaque 1077 Sigma H-8889) is layered under the diluted sample and centrifuged, 300 x g for 30 min. The mononuclear cell band is removed and washed two times in 1X PBS and once with 1% BSA PBS (CellPro Co., Bothel, WA). Mononuclear cells are counted

and CD34+ cells are selected using the Ceprate LC (CD34) Kit (CellPro Co., Bothel, WA) column. This fractionation is performed since all stem and progenitor cells within the bone marrow display CD34 surface antigen.

[0088] Cultures are set up in triplicate with a final volume of 1.0 mL in a 35 X 10 mm petri dish (Nunc#174926). Culture medium is purchased from Terry Fox Labs. (HCC-4230 medium (Terry Fox Labs, Vancouver, B.C., Canada) and erythropoietin (Amgen, Thousand Oaks, CA.) is added to the culture media. 3,000-10,000 CD34+ cells are added per dish. FLT3 receptor agonist proteins, in conditioned media from transfected mammalian cells or purified from conditioned media from transfected mammalian cells or *E. coli*, are added to give final concentrations ranging from .001 nM to 10 nM. Cultures are resuspended using a 3cc syringe and 1.0 mL is dispensed per dish. Control (baseline response) cultures received no colony stimulating factors. Positive control cultures received conditioned media (PHA stimulated human cells: Terry Fox Lab. H2400). Cultures are incubated at 37°C, 5% CO_2 in humidified air.

[0089] Hematopoietic colonies which are defined as greater than 50 cells are counted on the day of peak response (days 10-11) using a Nikon inverted phase microscope with a 40x objective combination. Groups of cells containing fewer than 50 cells are

referred to as clusters. Alternatively colonies can be identified by spreading the colonies on a slide and stained or they can be picked, resuspended and spun onto cytospin slides for staining.

Human Cord Blood Hemopoietic Growth Factor Assays

[0090] Bone marrow cells are traditionally used for in vitro assays of hematopoietic colony stimulating factor (CSF) activity. However, human bone marrow is not always available, and there is considerable variability between donors. Umbilical cord blood is comparable to bone marrow as a source of hematopoietic stem cells and progenitors (Broxmeyer et al., *PNAS USA* **89**:4109-113, 1992; Mayani et al., *Blood* **81**:3252-3258, *1*993). In contrast to bone marrow, cord blood is more readily available on a regular basis. There is also a potential to reduce assay variability by pooling cells obtained fresh from several donors, or to create a bank of cryopreserved cells for this purpose.

Methods

[0091] Mononuclear cells (MNC) are isolated from cord blood within 24 hr. of collection, using a standard density gradient (1.077 g/mL Histopaque). Cord blood MNC have been further enriched for stem cells and progenitors by several procedures, including immunomagnetic selection for CD14-, CD34+ cells; panning for SBA-, CD34+ fraction using coated flasks from

Applied Immune Science (Santa Clara, CA); and CD34+ selection using a CellPro (Bothell, WA) avidin column. Either freshly isolated or cryopreserved CD34+ cell enriched fractions are used for the assay. Duplicate cultures for each serial dilution of sample (concentration range from 1 pM to 1204 pM) are prepared with 1x104 cells in 1ml of 0.9% methylcellulose containing medium without additional growth factors (Methocult H4230 from Stem Cell Technologies, Vancouver, BC.). In some experiments, Methocult H4330 containing erythropoietin (FLT3) was used instead of Methocult H4230, or Stem Cell Factor (SCF), 50 ng/mL (Biosource International, Camarillo, CA) was added. After culturing for 7-9 days, colonies containing >30 cells are counted.

MUTZ-2 Cell Proliferation Assay

[0092] A cell line such as MUTZ-2, which is a human myeloid leukemia cell line (German Collection of Microorganisms and Cell Cultures, DSM ACC 271), can be used to determine the cell proliferative activity of flt3 receptor agonists. MUTZ-2 cultures are maintained with recombinant native flt3 ligand (20-100ng/mL) in the growth medium. Eighteen hours prior to assay set-up, MUTZ-2 cells are washed in IMDM medium (Gibco) three times and are resuspended in IMDM medium alone at a concentration of 0.5-0.7 x 10E6 cells/mL and incubated at 37°C and 5%CO_2 to starve the cells

of flt3 ligand. The day of the assay, standards and flt3 receptor agonists are diluted to two fold above desired final concentration in assay media in sterile tissue culture treated 96 well plates. Flt3 receptor agonists and standards are tested in triplicate. 50µl of assay media is loaded into all wells except row A. 75µl of the flt3 receptor agonists or standards are added to row A and 25µl taken from that row and serial dilutions (1:3) performed on the rest of the plate (rows B through G). Row H remains as a media only control. The starved MUTZ-2 cells are washed two times in IMDM medium and resuspended in 50µl assay media. 50µl of cells are added to each well resulting in a final concentration of 0.25 x 10E6cells/mL. Assay plates containing cells are incubated at 37°C and 5%CO_2 for 44hrs. Each well is then pulsed with 1µCi/well of tritiated thymidine in a volume of 20µl for four hours. Plates are then harvested and counted.

Transfected cell lines:

[0093] Cell lines, such as BHK or the murine pro B cell line Baf/3, can be transfected with a colony stimulating factor receptor, such as the human flt3 receptor which the cell line does not have. These transfected cell lines can be used to determine the activity of the ligand of which the receptor has been transfected.

EXAMPLE 1

Isolation of cDNA encoding flt3 ligand

Three flt3 ligand clones were amplified from human bone morrow poly A+ RNA (Clontech) using NCOFLT, HIND160, and HIND165 PCR primers (according to the manufacturer's suggested conditions). These amplified PCR products were gel purified and cloned into the BHK expression vector pMON5723 generating pMON30237 (NCOFLT + HIND160), pMON30238 (NCOFLT + HIND165), and a deletion clone pMON30239 (NCOFLT + HIND165). The deletion in pMON30239 is of amino acid residues 89 through 106 (the numbering of the residues is based on the sequence of native flt3 ligand as shown in Figure 5a and 5b).

EXAMPLE 2

Sequence rearranged flt3 ligand were constructed using several methods and linker types. The first set of constructs containing the linker peptide (SerGlyGlyAsnGly)(SEQ ID NO:46) (where X = 1, 2, or 3) with the breakpoints 39/40, 65/66, and 89/90 were made using a two step PCR process described by Mullins et al. in which the front half and the back half of each final

sequence rearranged molecule is made separately in the first PCR step, then the paired products of the first reaction step are combined in a second PCR step and extended in the absence of exogenous primers.

For example, to make the three 89/90 breakpoint precursor molecules with the SerGlyGlyAsnGly SEQ ID NO:46, SerGlyGlyAsnGlySerGlyGlyAsnGly SEQ ID NO:47, and SerGlyGlyAsnGlySerGlyGlyAsnGlySerGlyGlyAsnGly SEQ ID NO:48 amino acid linkers (pMON32326, pMON32327 and pMON32328 respectively), six initial PCR products were generated. The following primer pairs were used in the first step PCR reaction: a) 89For/L5B; b) 89For/L10B; c) 89For/L15B; d) 89Rev/L5A; e) 89Rev/L10A; and f) 89Rev/L15A. The identical approach was used to make pMON32321 (39/40 breakpoint, primer pairs 39For/L10B and 39Rev/L10A) and pMON32325 (65/66 breakpoint, primer pairs 65For/L5B and 65Rev/L5A) precursors. Except as noted below, all subsequent PCR reactions utilized the components of the PCR Optimizer Kit (Invitrogen) and amplification conditions according to the manufacturers suggested protocol. Reactions were set up as follows: 50 pmole of each primer, 10 ul of 5X Buffer B [300 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 75 mM (NH4)2SO4], 5 U Taq polymerase, and 100 ng of heat denatured DNA (in this example pMON30238) template were combined, and brought to 45 ul final

volume with dH_2O. Reactions were pre-incubated for 1-5 minute at 80°C, then 5 ul of 10 mM dNTP added to each reaction, and heat denatured for 2 minutes at 94°C prior to amplification in a Perkin Elmer model 480 DNA thermal cycler. Seven DNA amplification cycles were done under the following conditions: heat denature for one minute at 94°C, two minutes annealing at 65°C, followed by a three minute extension at 72°C. Twenty three additional cycles consisting of a one minute heat denaturation at 94°C followed by a four minute annealing/extension at 72°C were done, followed by a final 7 minute extension cycle at 72°C. With the exception of pMON32328, the PCR amplification products were run out on a 1.2% TAE agarose gel, and the appropriate size bands (the major amplification product) were excised and purified using Geneclean II (Bio 101). Samples were resuspended in 10 ul dH_2O. The amplification products for pMON32328 were purified directly using a Wizard PCR Clean UP kit (Promega), and DNA eluted in 50 ul dH_2O.

The method to construct the precursors of pMON32322 (39/40 breakpoint, primer pairs 39For/L5B and 39Rev/L5A) was modified by increasing the amount of template to 1 ug, and by changing the PCR amplification conditions as follows: six cycles of 94°C, 1 minute, 65°C for 2 minute, and 72°C for 2 1/2 minutes, followed

by 15 cycles of 94°C for 1 minute, 70°C for 2 minutes, and 72°C for 2 minutes, followed by a single 72°C extension cycle for seven minutes.

The second PCR step utilized the gel-purified precursors from the first PCR step as a combination of primer/template as follows: 5 ul each of each precursor molecule (i.e. for pMON32328 the PCR products from primer pairs 89For/L5B and 89Rev/L5A), 10 ul of 5X Buffer B, 5 U of Taq polymerase, and 24 ul dH_2O. The reactions were heated for five minutes at 80°C, 5 ul of 10 mM dNTP was added, and the reactions heat denatured for 94°C for two minutes. DNA amplification conditions were as follows: 15 cycles of 94°C for one minute, 69°C for two minutes, followed then by a three minute extension at 72°C. To allow for complete extension, the last cycle was followed by a single extension step at 72°C for seven minutes. The 80 deg incubation time was reduced to two minutes and the number of cycles was decreased to ten cycles for pMON32325 (PCR products 65For/L5B and 65Rev/L5A). PCR reaction products of the appropriate size were gel purified on a 1.2 % TAE agarose gel using Geneclean II. For pMON32322 (39For/L5B and 39Rev/L5A) the annealing temperature was reduced to 68°C, and the extension time reduced to two minutes. In addition, the PCR product was purified using a Wizard PCR Clean Up kit (Promega) according to the suppliers suggested protocol. The second PCR

step was modified for pMON32326 (PCR products of 89For/L15B and 89Rev/L15A) as follows. Three sets of PCR reactions were set up identically as above, except for the sample buffer type (either 5X buffer B, D, or J - PCR Optimizer Kit). Composition of buffers D and J differ from buffer B only by pH or [$MgCl_2$]. The [$MgCl_2$] for buffer D is 3.5 mM, whereas the pH of buffer J is 9.5. The protocol was modified by increasing the number of PCR cycles 20, and 15 ul aliquots were withdrawn at the end of cycles 10, 15 and 20. Five uls of each aliquot timepoint were analyzed for the presence of amplified material on a 1.2% TBE agarose gel. The remainder of the buffer B, D, and J PCR reaction mixtures were pooled and subsequently purified using the Wizard PCR Clean Up Kit protocol. The DNA was eluted in 50 ul dH_2O.

The purified samples from the second step PCR reaction were digested with NcoI/HindIII using one of two standardized digestion conditions. For Geneclean II purified samples, 10 ul of DNA were digested in a 20 ul reaction with 7.5 U each of NcoI/HindIII for two hours at 37°C, and gel purified on a 1.1% TAE agarose gel again with Geneclean II. Ligation-ready samples were resuspended in 10 ul dH_2O. For pMON32322, 20 ul of sample was digested in a 50 ul reaction volume with 20U each of NcoI and HindIII for 3 hour at 37°C. 0.1 volume 3M NaOAc (pH 5.5) and

2.5 volume of EtOH were added, mixed, and stored at -20°C overnight. The DNA was recovered by pelleting for 20 minutes at 13,000 rpm @ 4°C in a Sigma Mk 202 microfuge. The DNA pellet was rinsed with chilled 70% EtOH, lyophilized, and resuspended in 10 ul dH_2O.

EXAMPLE 3

An alternate approach was used to construct pMON32320 (39/40 breakpoint, fifteen amino acid linker), pMON32323 (65/66 breakpoint, fifteen AA linker), and pMON32324 (65/66 breakpoint, ten amino acid linker). New primers (L15C, L15D, L15E) were designed to incorporate BamHI restriction site in the primer that was inframe to allow cloning into the BamHI site and maintain the proper reading frame. PCR reaction conditions for the first step were performed identically to that described for pMON32322, except that the following set of primer pairs were used: 65For/L15D and 65Rev/L15E (pMON32324); 39For/L15D and 39Rev/L15C (pMON32320); and 65For/L15D and 65Rev/L15C (pMON32323). The PCR reaction products were purified using a Wizard PCR Clean Up kit as described, and eluted in 50 ul dH_2O. Samples were digested with either NcoI/BamHI (39For/L15D and 65For/L15D) or BamHI/HindIII (39Rev/L15C, 65Rev/L15C, and 65Rev/L15E). Restriction digests were performed as follows: 10 ul of purified

PCR reaction products, 3 ul of 10X universal restriction buffer, 15 U of either NcoI or HindIII, 15 U of BamHI, in a final reaction volume of 30 ul. Reactions were incubated for 90 minutes at 37°C, and the PCR products gel purified on a 1.1% TAE agarose gel using Geneclean II. Ligation-ready DNA was resuspended in 10 ul dH_2O.

Inserts were ligated to NcoI/ HindIII digested pMON3977 (BHK mammalian expression vector) that had been treated with shrimp alkaline phosphatase (SAP) either in a three way (pMON32320, pMON32323, or pMON32324) or a two way (pMON32321, pMON32322, pMON32325, pMON32326, pMON32327 and pMON32328) ligation reaction as follows: 2.5 ul of insert (2 ul of each primer pair amplicon for pMON32320, pMON32323, and pMON32324) was added to 50 ng of vector in a ten ul reaction using standard ligation conditions. Two ul of each reaction was transformed with 100 ul of chemically competent DH5α cells (Gibco/BRL) following the manufacturers suggested protocol. Twenty five ul and 200 ul aliquots were plated out on LB plates containing 50 ug/mL ampicillin and incubated overnight. Isolated colonies were picked and DNA prepared from 50 mL overnight cultures using Qiagen DNA midiprep kits. DNA was quantitated by absorbance at A260/A280, and verified for correct insert size by agarose gel electrophoresis following digestion of 1 ug template with NcoI/HindIII

restriction endonucleases. Samples containing inserts of the predicted size were sequenced in both orientations using vector-specific primers using an automated fluorescent DNA sequencer model 373A (Perkin Elmer ABI). Sequencing reactions were done in 20 ul reaction volumes using a Perkin Elmer model 480 DNA thermal cycler as follows: one ug of template, 3.2 pmole primer, 1 ul DMSO, 9.5 ul Taq terminator dyedeoxy premix (Perkin Elmer ABI) were combined, and subjected to 25 cycles of sequencing amplification as follows: 30 seconds at 94°C, 15 second annealing at 50°C, followed by a four minute extension cycle at 60°C. Samples were purified using Centri-Sep spin columns (Princeton Separations) following the manufacturers suggested protocol, lyophilized, and submitted for sequence analysis. Samples containing the predicted amino acid sequence were selected for analysis and assigned pMON numbers.

EXAMPLE 4

A similar approach used to construct pMON32320, pMON32323, and pMON32324 was utilized to introduce the second linker type (SerGlyGlySerGly)X where x = 2 or 3, into two sequence rearranged flt3 receptor agonists containing the 39/40 breakpoint (pMON32348 and 32350). The primer pairs were as follows: for pMON32348 the combinations of 339For2/339Rev3 and 339Rev2/339-10For3 and for pMON32350 the combinations of 339For2/339Rev3 and 339Rev2/339-

15For3 were used to create three PCR amplification products. Each PCR amplification was set up as follows: to 100 ng of heat denatured pMON32320, 50 pmole of each primer pair, 10 ul of 5X Buffer B, 5 U of Taq polymerase and dH_2O was added to a final volume of 45 ul. Reactions were pre-incubated as described before. Fifteen amplification cycles were done under the following conditions: heat denature at 94°C, one minute, followed by a two minute annealing step at 70°C, and a three minute extension at 72°C. After the last cycle, a single 72 deg extension step of 7 minutes was done. The PCR amplification products of primer pairs 339For2/339Rev3, 339Rev2/339-10For3, and 339Rev2/339-15For2 were purified using a Wizard PCR Clean Up kit (Promega), and eluted in 50 ul dH_2O. NcoI/BamHI digests for the 339For2/339Rev3 primer pair as follows: 8 ul of DNA template was mixed with 2 ul universal restriction buffer and 10 U each of NcoI and BamHI in a 20 ul reaction volume, and incubated for 90 minutes at 37°C. The digestion products was purified using the Geneclean II direct purification protocol, and ligation ready DNA resuspended in 10 ul dH_2O. The restriction digests and subsequent purification for the 339Rev2/339-10For3 and 339Rev2/339-15For2 amplification products were done identically as described for the 339For2/339Rev3 amplicon, except that 10 U

of HindIII was substituted for NcoI. Standard ligations were done by adding to 50 ng NcoI/HindIII/SAP-treated, gel purified pMON3977, 0.5 ul 339For2/Rev3 amplicon, 1 ul of either 339Rev2/339-10For3 (pMON32348) or 339Rev2/339-15For3 (pMON32350) amplicons, 5U T4 DNA ligase, and 1 ul 10 X ligase buffer in a 10 ul reaction volume for 60 minutes at ambient temperature. Subsequent steps leading to final DNA sequence confirmation were done as described above.

EXAMPLE 5

A third type of linker, with a variable (GlyGlyGlySer) (SEQ ID NO:38) repeat motif, was incorporated into another set of sequence rearranged flt3 receptor agonists from modularly constructed templates. These linker lengths were;

6 AA linker (GlyGlyGlySerGlyGly SEQ ID NO:51),
7 AA linker (GlyGlyGlySerGlyGlyGly SEQ ID NO:52),
10 AA linker (GlyGlyGlySerGlyGlyGlySerGlyGly SEQ ID NO:53),
13 AA linker (GlyGlyGlySerGlyGlyGlySerGlyGlyGlySerGly SEQ ID NO:54),
15 AA linker (GlyGlyGlySerGlyGlyGlySerGlyGlyGlySerGlyGlyGly SEQ ID NO:55); and
21 AA linker (GlyGlyGlySerGlyGlyGlySerGlyGlyGlySerGlyGlyGlySerGlyGly GlySerGly SEQ ID NO:56) amino acid residues. These modular templates, each comprising a dimer of hflt3 ligand separated by a BamHI-containing linker of unique length, were constructed as

follows. Six intermediate PLASMID templates, FL3N, FL7N, FL11N, FL3C, FL4C, and FL10C, were constructed by PCR using paired primers and pMON30238 as template using cycling conditions similar to those employed for pMON32322. Per reaction, 50 pmole of each primer was added to 100 ng of heat-denatured template and the reactions assembled as described for pMON32322. Cycle conditions were as follows: seven cycles of 94°C, one minute; two minutes at 65°C, and 2.5 minutes at 72°C; followed by ten cycles of one minute at 94°C, two minutes at 70°C, and 2.5 minutes at 72°C. A single seven minute extension at 72°C completed the cycling reactions. The primer pairs used to construct each intermediate were; N-term/FLN3 (FL3N); N-term/FLN7 (FL7N); N-term/FLN11 (FL11N); C term/FLC3 (FL3C); C-term/FLC4 (FL4C); and C-term/FLC10 (FL10C). The PCR amplification products were purified with Wizard PCR Clean Up kits (Promega) and eluted in 50 ul dH_2O. Purified DNA for the first subset, FL3N, FL7N, and FL11N, were digested with NcoI/BamHI, gel purified as described previously, and ligated to NcoI/BamHI/Sap-treated pSE420 vector DNA (Invitrogen). Intermediate templates of the second subset, FL3C, FL4C, and FL10C, were constructed in an identical manner except HindIII was utilized instead of NcoI. Subsequent steps leading to final DNA sequence confirmation were done as described above.

EXAMPLE 6

To make the next six templates, the two subsets of intermediates in pSE420 were digested with either NcoI/BamHI (FL3N, FL7N, FL11N-subset 1) or BamHI/HindIII (FL3C, FL4C, FL10C-subset 2) and gel purified using Geneclean II as described previously. One intermediate amplicon from each subset were ligated to NcoI/HindIII/SAP-treated pMON3977 per reaction and transformed in DH5α cells as described previously using the following combinations to generate specific linker lengths: six AA linker (FL3N and FL3C), seven AA linker (FL3N and FL4C), ten AA linker (FL7N and FL3C), thirteen AA linker (FL3N and FL10C), fifteen AA linker (FL11N and FL4C), and 21 AA linker (FL11N and FL10C). DNA was prepared 50 mL overnight cultures from single colonies from each of the six combination as described above, analyzed for correct insert size by NcoI/HindIII restriction analysis, and used as template.

Primer pairs 39For/39Rev (39/40 breakpoint); 65For/65Rev (65/66 breakpoint) and 89For/89Rev (89/90 breakpoint) were used to PCR amplify each templates as described for pMON32322, except 75 pmole of each primer was used. Amplification conditions were modified as follows: six cycles of 94°C for one minute, 2 minutes at 70°C, 2.5 minutes at 72°C; followed by nine cycles of 94°C for one minute, and three minutes at 72°C. After the last cycle,

a final extension of six minutes at 72°C allowed ample time for full extension of products.

Samples were purified using a Wizard PCR Clean Up kit as described, and double digested with NcoI/HindIII. These amplification products were purified again using a Wizard PCR Clean Up kit. In addition, all six different linker length molecules for the 39/40 breakpoint were cloned into NcoI/HindIII/SAP-treated pMON3977 as single proteins (pMON32365, pMON32366, pMON32367, pMON32368, pMON32369 and 32370). Subsequent steps leading to final DNA sequence confirmation were done as described above.

EXAMPLE 7

Additional sequence rearranged Flt3 ligands were constructed using the dimer template intermediates previously described. For sequence rearranged Flt3 ligands having the fifteen amino acid linker $(GlyGlyGlySer)_3GlyGlyGly$ SEQ ID NO:55, the dimer intermediates Flt4C.seq and Flt11N.seq were used as the template in the PCR reaction. Five new breakpoints corresponding to Flt3 ligand amino acid residues 28/29, 34/35, 62/63, 94/95, and 98/99, were constructed using a PCR based approach using a PCR Optimizer kit (Invitrogen) and the following primer pairs; FL29For/FL29Rev, FL35For/FL35Rev, FL63For/FL63Rev, FL95For/FL95Rev, FL99For/FL99Rev. Amplification conditions were as follows: seven

cycles of 94°C for 1′, 62°C for 2′, and 2.5′ at 70°C; twelve cycles of 94°C for 1′, 68°C for 2′,and 70°C for 2.5′; followed by a final cycle of 7′ at 72°C. PCR products corresponding to the predicted insert size were digested to completion with NcoI and HindIII, and gel purified as described previously using Gene Clean II (Bio 101) following the manufacturers suggested protocol. Samples were resuspended in 10 ul final volume with dH_2O. Inserts were cloned as single genes into the mammalian expression vector pMON3977 (NcoI/HindIII/SAP treated) and designated pMON35712, pMON35713, pMON35714, pMON35715, pMON35716, pMON35717, pMON35718 respectively.

[0094] Additional techniques for the construction of the variant genes, recombinant protein expression , protein purification, protein characterization, biological activity determination can be found in WO 94/12639, WO 94/12638, WO 95/20976, WO 95/21197, WO 95/20977, WO 95/21254 and WO 96/23888 which are hereby incorporated by reference in their entirety.

[0095] All references, patents or applications cited herein are incorporated by reference in their entirety as if written herein.

[0096] Various other examples will be apparent to the person skilled in the art after reading the present disclosure without departing from the spirit and scope of the invention. It is

intended that all such other examples be included within the scope of the appended claims.